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Title:
The adaptive value of males in simple eusocial insect societies

The Adaptive Value of Males in Simple Eusocial Insect Societies

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A dissertation submitted to the University of Bristol
in accordance with the requirements for award of
the degree of Doctor of Philosophy in the
Faculty of Science (July 2018)

Word Count: 69,733

The adaptive value of males in simple eusocial insect societies

Abstract

In the Hymenoptera (ants, bees, and wasps), female inclusive fitness is maximised directly by reproducing or indirectly through helping kin. By contrast, males have mainly evolved to disperse and mate, limiting fitness opportunities to direct reproduction. However, there are reports across the social Hymenoptera of natal males helping, by distributing food to brood, as well as defending and thermoregulating nests. It is not fully understood under what circumstances male help, how it is regulated, or whether helping carries indirect fitness benefits. Here, the hypothesis is tested that males are more than just 'flying sperm', and may have diversified pathways to maximising inclusive fitness. In achieving this aim, two tropical simple eusocial Polistinae paper wasps are studied, *Polistes canadensis* and *Polistes lanio*. First, through discovery and validation of new single-nucleotide polymorphism (SNP) markers, it is shown that siblings on the nest are fully related; suggesting indirect fitness opportunities for brothers. Second, using captive studies of *P. canadensis*, it is found that females are long-lived; suggesting short-term male investments have the potential to be assured by sisters after males depart. Third, males of *P. lanio* are delayed dispersers: in field studies males remained on the natal nest whilst sexually maturing; eventual dispersal was regulated by juvenile hormone. Delayed dispersal with immaturity suggests a life history phase in which direct fitness gains are not achievable for adult males. Finally, by experimentally offering food to natal *P. lanio* males in field trials, males were shown to help by distributing food to brood; but this effort diminished with age, suggesting male helping is temporary. In conclusion, it is proposed that males have the potential to exploit two different fitness strategies in their life, of a young helper that may benefit from indirect fitness gains and old reproductive that invests in mating and direct fitness.

Acknowledgements

First and foremost, I would like to thank the wasps. I have lost count of how many times I have been stung now, but I am sure I deserved each sting. I'd also like to thank my supervisors Seirian Sumner and Andy Radford, without their help (and patience) this study would have not been possible. The University of Bristol has provided support and encouragement throughout, and I would like to thank the School of Biological Sciences and its occupants. In addition, University College London took me in after my supervisor moved, and treated me as one of their own. Whilst in Trinidad, the University of the West Indies opened their doors to me and provide immense help without question when I showed up lost and confused on the hunt for wasps, and have been instrumental in this study (thanks for the last minute dry ice). Part of this project used samples collected from Panama, and for that I am grateful to the Smithsonian Tropical Research Institute. This study was primarily funded by the Natural Environment Research Council (NERC), with additional funding from the Genetics Society (fieldwork grant), and Bristol University's Alumni Foundation (conference travel grant). Thank you to Innes Cuthill and Elli Leadbeater for examining my viva.

The 'Sumner lab group' has been my colleges and friends for the past four years, and I would like to thank Alessandro Cini, Ben Taylor, Chris Wyatt, Sam Duckerin, and Sandra Moreno. A few people in the group need a descriptive shout-out: Patrick Kennedy, you beat my 'being stung' count by far, for that you have my respect; Peter Graystock, thanks for teaching me how to use a pipette; Adam Devenish, our coffee/pub breaks are a cornerstone of this study; Daisy Taylor, thanks for taking me to hospital for apparent vampire bat induced rabies. Last and certainly not least, Emily Bell this all started when I turned up one day in Panama as your field assistant and escalated into this study (time will tell if I am thankful for that), you've been a best friend and your support has been unparalleled. I would also like to thank everyone else at both Bristol University and UCL, especially Calum McIntosh, Charlotte Bickler, Chris Langridge, Dan Jue Lan, Henry Ferguson-Gow, Jon Bridle, Paul Parsons, Brett Marshbanks, Barney Wharam (always smiling), and no other than Jeroen "J" Martins (thanks for lessons in solution dilution at 12 o'clock at night).

Paramount in the field has been the help of Daniel Fabbro, Joshua Valverde (funded by the Genetics Society), Liam Crowley (NERC), and Sam Morris (NERC), thanks for dealing with the rain and machete wielding men. At STRI, thank you to William Wcislo and Donald Windsor for their help. At UWI, I would like to thank Chris Starr and Mike Rutherford for their knowledge and insight into the local wasps. In Arima Valley, Ricki, Baldeo, Mashesh, and their families, thanks for letting us into your homes. In addition, thank you to Andrew and Calum Stephenson for their knowledge of the island and help. Also, the 'gupsters' of Arima Valley, for showing me another side of Trinidad that wasn't chicken sheds and abandoned buildings (also the 'other gupsters' hiding up in Simla, Kevin Horng, Ronnie Hernandez, and Sean Ehlman). A descriptive shout-out to Toby Hector, no one else appreciates puncheon as much as you; and Ashley Hedrick, you've had to deal with me the most, and have supported me the most, your funnelling of beer, nachos, and that food you make which I cannot pronounce has been valued more than anything. Lastly, the occupants of 'home on the hill', thanks for all the stag.

Special thanks to my parents, Fran Solich & Steve Southon.

Permits

Panama, 2013: ANAM research permit SE/A-55-13 (Emily Bell & Seirian Sumner)
Trinidad & Tobago, 2013: Wild Life Section, Special Game License (Seirian Sumner)
Trinidad & Tobago, 2014: Wild Life Section, Special Game License (Robin Southon)
Trinidad & Tobago, 2015: Wild Life Section, Special Game License (Robin Southon & Daisy Taylor)

Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Chapter 1. General Introduction

1.1. The evolution of sociality and altruism

The rise of sociality has been described as one of the major transitions in evolution, in which conspecific individuals aggregated to form long lasting social groups (Maynard Smith & Szathmáry, 1995). Grouping with conspecifics may provide benefits of reduced risk from predation, facilitation of movement in the environment, shared thermoregulation, social learning, and the ability to tackle complex food sources. However, group living may also have negative consequences, of increased competition (both resource and mate), increased disease susceptibility, exploitation from other group members, increased risk of cannibalism, and inbreeding (reviewed in Barnard, 2004). How and why groups are formed, and what maintains group stability, is one of the key questions in the study of animal behaviour.

Interactions between individuals within groups dictate what types of relationships bind members together. Behavioural interactions between conspecifics can be classified as: 'selfish', the behaviour benefits the actor but has negative consequences on the recipient; 'mutually beneficial', the behaviour benefits both the actor and the recipient; 'altruistic', the behaviour has a negative consequence for the actor, but a positive effect on the recipient; and 'spiteful', the behaviour has negative consequences for both the actor and recipient (whether spite is a form of indirect altruism is questionable, as it may benefit a third party – West & Gardner, 2010) (Hamilton, 1964; Krebs & Davies, 1993; West et al., 2007). The strength of effect in interactions is not always equal; for example, if an interaction has a low cost for the actor but high benefit to the recipient, this could be described as 'weakly altruistic' (Gadagkar, 1993). Additionally, interactions are not necessarily performed directly for the recipient, for example if a positive consequence for a recipient was a 'by-product' of a selfish action (Clutton-Brock, 2009). Relationships between group members may encompasses a range of behavioural interactions, but some interactions are known to define certain group structures, such as if the behaviour is critical to the maintenance of complex sociality.

Altruistic interactions between conspecific group members can be observed across the animal kingdom, but why should an individual put itself at a disadvantage to benefit another? If temporal lifetime benefits are uncoupled, then some altruistic acts can be viewed as reciprocal (Trivers, 1971). For example, many primates such as grivets *Cercopithecus aethiops* and crab-eating macaques *Macaca fascicularis* will come to the aid of those that had previously sacrificed time and energy to groom them (Seyfarth & Cheney, 1984; Hemelrijk, 1994;

reviewed in Schino & Aureli, 2009). Non-reciprocal altruism, whereby direct negative effects to the actor are not balanced out later in life, is often observed in eusocial species. In the Wilsonian sense, eusociality is characterised by societies in which a subset of group members are devoted to extreme altruism, sacrificing their own reproductivity to help others reproduce. Further, eusocial societies have reproductive division of labour, with overlapping generations, and cooperative care of young (Wilson, 1971; Wilson, 1975). For example: colonial Aphidoidea aphids produce sterile soldiers; highly related social amoeba form groups in which some members sacrifice themselves to form a 'stalk' – allowing others to migrate to new areas; siblings protect dependant brood in the ambrosia beetle *Xyleborinus saxesenii* (Biedermann & Taborsky, 2011); and insect societies of the Hymenoptera (ants, bees, and wasps) and Isoptera (termites) possess effectively sterile altruistic worker castes (Wilson, 1971; Gadagkar & Bonner, 1994; Thorne, 1997; Strassmann et al., 2000; Abbot et al., 2001; Gilbert et al., 2007). Some of the clearest examples of eusociality are found in these insects with castes. Darwin (1859) wrote that insect castes presented a difficulty in his theory of natural selection, as the parents of sterile workers would be expected to pass on traits that encourage survivability and reproduction. However, Darwin speculated altruism and reproductive loss may be linked to a benefit of helping kin within the group (Ratnieks et al., 2011).

Inclusive fitness theory, proposed by Hamilton (1964), explains how altruistic actions may indirectly benefit the actor. Inclusive fitness is the sum of both direct and indirect fitness, direct fitness being the fitness gained by passing on genes to offspring, whilst indirect fitness is gained when an individual helps a relative with shared genes reproduce. Hamilton's rule proposes the equation $rb > c$, in which r is the genetic relatedness between the actor and recipient; b the benefit to the recipient; and c the cost to the actor. In an interaction, if the relatedness and benefit to the recipient is greater than the cost, then altruistic acts may be selected for (Hamilton, 1964). A process termed kin selection by Maynard Smith (1964). Though true tests of Hamilton's rule are difficult, there is empirical support for it (reviewed in Bourke, 2014). Hamilton's rule has defined the study of social living, but it does make broad predictions and excludes biological complexity (Queller, 1991; Queller, 1992), and has gathered controversy (Nowak et al., 2010; Nowak et al., 2017). Perhaps much of this controversy results not from Hamilton's general rule, but its application to the haplodiploid hypothesis in eusocial Hymenoptera; specifically, that extreme cases of altruism in these societies arises from unusually high relatedness between sisters due to haplodiploid sex determination (Hamilton, 1964; Queller & Strassmann, 1998; Alpedrinha et al., 2013).

1.2. Altruism in female Hymenoptera

In the eusocial Hymenoptera (ants, and some bees and wasps), an extreme form of altruism is present in female nestmates which forgo reproduction to help raise related sisters; these females are effectively a sterile 'worker caste' that specialise in indirect fitness gains. On the other end of the phenotypic spectrum, is a reproductive 'queen caste', that specialises in egg production and hence gaining direct fitness (Wilson, 1971; Crespi & Yanega, 1995; Bourke & Franks, 1995). This reproductive division of labour can be demonstrated clearly in eusocial Hymenoptera where castes are committed and fixed during development. For example, in Formicidae ants such as the leaf-cutter *Atta sexdens*, nonbreeding female workers occupy roles of nest defenders, foragers of nest resources, and nurses which tend to the fungus garden and brood; in contrast, within each nest is also a queen, that solely controls reproductive output and avoids performance of other labour tasks (Wilson, 1980).

The extent of caste commitment in Hymenoptera varies between species and social structures. In simple eusocial species (also termed primitively or basal eusocial) castes are flexible in adulthood, females may switch between being a nonbreeding helper/worker or reproducing breeder/queen (to some extent, see Tsuji & Tsuji, 2005; Jeanne & Suryanarayanan, 2011). In complex eusocial species (also termed advanced eusocial) castes are fixed during development and therefore inflexible in adulthood, individuals are committed to a lifetime of worker or queen behaviour (Crespi & Yanega, 1995). In this thesis, the terms simple and complex eusocial are used to broadly allow easier comparisons in the literature. However, it has been suggested that many simple eusocial hymenopteran species may be no more different from obligatory cooperative breeders, in which a dominant individual possesses a reproductive monopoly with subordinate helpers who retain reproductive capacity and may later become breeders themselves (Boomsma, 2007; Field & Leadbeater, 2016). Recently, Boomsma & Gawne (2017) proposed to redefine the term eusociality in the Hymenoptera: they distinguished between facultative or obligate cooperative breeding groups (with facultative or partly obligate caste commitment respectively, e.g. *Polistes* paper wasps) and superorganisms (obligate caste commitment, unmated workers, and domestication of the queen caste by the workers, e.g. the honeybee *Apis mellifera*). These superorganisms may represent the binding of individual organisms into a new unit of individuality, akin to the evolutionary transition of single cell units becoming multicellular organisms (Wheeler, 1911; Kennedy et al., 2017). Irrespective of classification, the social Hymenoptera possess some of the most complex forms of sociality amongst the Eukaryota.

Societies of Hymenoptera are often thought as female-centric, such that division of reproductive labour is only between female castes. Hymenoptera are haplodiploid organisms, in which the sex of the offspring is usually reliant on single locus complementary sex determination: females are heterozygous, the result of diploid fertilised eggs; males are hemizygous haploids resulting from unfertilised eggs (Evans et al., 2004; van Wilgenburg et al., 2006). Hamilton (1964) predicted that because haplodiploidy results in full-sisters being on average more related to each other than their own offspring, as they are guaranteed to share the haploid father's chromosome (coefficient of relatedness: offspring, r = 0.50; full-sister, r = 0.75), then a sterile female worker caste may evolve because relatedness and benefit to sisters outweighs the cost of forgoing reproduction. Despite this prediction, relatedness asymmetries alone cannot account for a female worker caste (Trivers & Hare, 1976). Assuming a simple model of a hymenopteran group with a single singly mated queen and her offspring (relatedness calculated as 'life-for-life': male genes passing only to daughters, and females to both sexes, giving differing weighting to diploid/haploid sex values – Bourke & Franks, 1995): with an equal sex ratio, females are on average equally related to siblings in the group (full-sisters, r = 0.75; brothers, r = 0.25; average relatedness with 1:1 sex ratio, r = 0.50) as they are to their own offspring (daughters and sons, r = 0.50). This averaging effect of lower relatedness to brothers cancels out the benefit of high relatedness to full-sisters, and stems from female ancestral monogamy and the preferred sex ratio of the dominant reproductive being equal (Hughes et al., 2008; Ross et al., 2013). Ancestral monogamy is likely at the evolutionary transition to eusociality, as it allows any small kin selected benefits associated with caste commitment (such as if nonbreeding helpers gave groups higher productivity) to manifest over time (Boomsma, 2007; Hughes et al., 2008; Boomsma, 2009).

If worker caste females could control the sex ratio towards being female-biased, then haplodiploidy could contribute to the evolution of female helpers (Trivers & Hare, 1976). However, this is not a viable argument for two reasons. First, simultaneous production of more females than males would increase the value of male offspring (Gardner et al., 2011). Second, split sex ratio theory predicts that when offspring sex ratio is controlled by nonbreeding female workers: groups with high relatedness asymmetries should specialise in production of females; and groups with low asymmetries (such as if the group contains multiple related reproductives) should specialise in production of males (Boomsma, 1991; Meunier et al., 2008). Despite split sex ratios being present in some eusocial species, in the context of an evolutionary transition towards fixed caste commitment, traits allowing for nonbreeding female worker control of the sex ratio (such as worker policing of male eggs) likely evolved after lifetime caste commitment – and hence split sex ratios cannot account for the evolution of a female worker (Gardner et al., 2011).

The current dominant explanations for the evolution of female workers castes are thus. Proximate explanation: uncoupling of ancestral maternal care traits towards female alloparental care between siblings (Linksvayer & Wade, 2005). Alloparental care is defined as the act of helping another individual's offspring, which increases the indirect fitness of the actor (Wilson, 1975). Current theory speculates that if the ancestral female phenotype could switch between being a reproductive and a maternal carer of young, then the loss of this plasticity would result in a philopatric female that remained on natal nest and helped raise siblings (West-Eberhard, 1987; Queller & Strassmann, 1998; Linksvayer & Wade, 2005; Ross et al., 2013; Toth et al., 2007). Ultimate explanation: females are 'life insurers' (Queller & Strassmann, 1998). Females possess many traits that allow them to hunt for and care for dependent offspring, but they must leave the safety of the nest to gather resources to do so. Consequently, a solitary female is at a high risk of losing investment in dependent brood if she dies whilst foraging. But in a group of females with overlapping lifespans, in which one generation cares for brood and subsequently has its offspring cared for by the next generation, then fitness returns are assured (Gadagkar, 1990; Queller & Strassmann, 1998). Life insurance theory compliments inclusive fitness theory, but removes the requirement that the relatedness component of r (in Hamilton's rule of $rb > c$) must be very high (such as in the Haplodiploid hypothesis, in which $r = 0.75$ is key). Specifically, by retaining helpers at the nest, the chance of a mother's brood being reared to adulthood is increased as the nest is not left unprotected whilst the mother forages, or untended if the foraging mother dies. Foragers in social insect societies are subjected to high mortality rates such that there is a high probability that a forager will die before her investment (i.e. the brood she has tended) is realised (i.e. the brood reach adulthood). If the fitness of the part-raised brood can be assured by other nestmates, then the payoffs from investing in indirect fitness may be enough for a female to choose to stay as a helper rather than disperse and nest independently, where she would potentially gain direct fitness. Further, by staying rather than dispersing, the nest productivity (number of brood raised) is increased by multiple foraging adults. For philopatric helpers, if relatedness r is greater than 0 (as there must be a minimum fitness component), then any benefit b from nest productivity that is greater than the cost c should select for cooperation (Strassmann & Queller, 2007). Transition towards castes: females of species must go through three evolutionary transitions to reach a society with fully committed castes, being (1) specialisation of breeders, (2) the evolution of some offspring with helping traits and reduced fecundity, and (3) the evolution of discrete reproductive division of labour, individuals are either committed to a lifetime as a worker or queen caste (Boomsma & Gawne, 2017). However, these explanations focus on the female sex being the driving force of sociality in the Hymenoptera, and this is because the male sex is often regarded as having little role in societies beyond being a reproductive extension of the queen caste (Hölldobler & Wilson, 1990).

1.3. The lack of helping behaviour in Hymenoptera males

In comparison to their altruistic sisters, hymenopteran males are often perceived as selfish faineants within the societies to which they belong (Aristotle, c. 350BC; Wilson, 1971; Wilson, 1975). Culturally, the view of the lazy male drone and a ruling queen with her hard-working daughters was ignored in many texts of the 16th to 18th century, Shakespeare himself an amateur apiarist describing bees as having "a king and officers of sorts" (Prete, 1991; Grinnell, 2016). The modern (and more correct) perception of males in hymenopteran societies has swung the other way, Hölldobler & Wilson (1994) summarising the view of males as "Males are thus drones in the original, Old English sense of the word: *drons*, parasites who live on the labor of others. They are also drones in the modern technological sense, flying sperm-bearing missiles constructed only for the instant of contact and ejaculation. Whilst on the nest, however, they are totally dependent on their amazon sisters and apparently are tolerated solely for their ability to transmit the colony's genes." Helping in hymenopteran societies by fathers and brothers, and by extension a male worker caste, is expected to be rare or absent (Hamilton, 1972; Starr, 1985b; Beani et al., 2014) (Note: While some parasitoid wasps produce male and female polyembryonic soldiers, due to their clonal nature evolutionary pressures are likely extremely different from that of normal breeding Hymenoptera, see Giron et al., 2004; Gardner et al., 2007). To explain this rarity of male helping, four main interconnecting factors in hymenopteran male evolutionary history have been proposed.

1.3.1. Absence of male hymenopteran helpers: lack of ancestral paternal care

The first potential explanation for why male helping behaviour is rare in the Hymenoptera relates to the lack of ancestral traits associated with paternal care. Parental care, in which one or both sexes provide post- natal or oviposition care (e.g. direct care of the offspring outside the womb) to offspring is a major phenomenon of social living (Wilson, 1975). Care of offspring may be reflected in behaviours where parents provide dependent young with food, defence from predation, or thermoregulation (Clutton-Brock, 1991). By providing care to developing young, parents may invest in increasing the fitness of individual offspring (K-selection); this contrasts with a strategy of little investment in the individual, and instead spreading net fitness among many offspring (r-selection) (Pianka, 1970; Stearns, 1976). Which sex cares can be attributed to various selection pressures, of fitness payoffs between the sexes, sexual conflict, and differences in ecological conditions (Maynard Smith, 1977; Wade & Shuster, 2002; Alonzo & Klug, 2012). Parental care may be evolutionarily redirected as alloparental care. This redirection of ancestral parental care is apparent in the female castes of Hymenoptera (West-Eberhard, 1987; Queller & Strassmann, 1998; Linksvayer & Wade, 2005; Ross et al., 2013; Toth et al., 2007).

In eusocial species with ancestral traits associated with bi-parental care, extended alloparental care in both sexes and hence a male worker caste is apparent. Examples of a male work caste can be found across multiple independent origins of eusociality (we list the eusocial Thysanoptera gall thrips as a separate unique case, see 1.3.5. Absence of male hymenopteran workers: other causations). In the simple eusocial naked *Heterocephalus glaber* and Damaraland *Fukomys damarensis* mole-rats (both two independent linages of eusociality), nonbreeding male and female helpers equally perform tasks to the benefit of kin, including: nest building; foraging by digging tunnels, transporting food, and recruitment; keeping neonates warm; carrying neonates away from danger; and in larger bodied *H. glaber* nonbreeding helpers, high risk defence behaviour against predators and conspecific intruders. Simple eusocial mole-rat groups typically contain both a resident queen and one to three breeding males, hence ancestral bi-parental care may be predicted by paternal presence (Faulkes & Abbott, 1991; Faulkes et al., 1991; Jarvis & Bennett, 1993; Bennett, 1994; O'Riain & Jarvis, 1997; Cooney, 2002). The simple eusocial *Synalpheus* shrimp species, for example *Synalpheus regalis* and *Synalpheus rathbunae*, also possess male helpers. Large mature (but nonbreeding) males of *S. regalis* will engage in active nest defence within the group's sponge host (in which they shelter and feed in), by snapping at heterospecific and conspecific intruders. In addition to defending the nest from predation, male snapping behaviour may maintain genetic relatedness within the group, as unrelated individuals are blocked from joining and diluting relatedness. Offspring in simple eusocial groups of *Synalpheus* share relatedness via a single mother that controls reproduction, who is typically mated to a single breeding male. However, whether this mated male is a full resident of the group or not is unknown (Duffy, 1996; Duffy et al., 2002; Duffy, 2007; Ďuriš et al., 2011; Hultgren et al., 2017). Perhaps the most distinct example of a male worker caste is in the simple and complex eusocial termites. Nonbreeding male helpers in termite nests perform an array of tasks associated with indirect fitness potential, such as nest building, brood care, and foraging (though performance of tasks vary within and between sexes across species). Termite nests may contain long lived and continuously breeding reproductive queens and kings, with bi-parental care present during the nest founding period in most species, allowing a pathway for the evolution of male alloparental care and castes (Shellman-Reeve, 1990; Higashi et al., 1991; Rosengaus & Traniello, 1991; Thorne, 1997; Shellman-Reeve, 1999; Johnstone & Wheeler, 2007). However, an exception in sharing of helping-tasks between the sexes is in the defensive termite solider caste, which is typically female-biased and possibly has independent evolutionarily origins from other helping tasks (Muller & Korb, 2008). In termite soldiers, selection for female fecundity has increased female size in comparison to males, and this size increase may predispose females to soldier roles over males (Matsuura, 2006). Ultimately, the evolution of alloparental care and castes in males may be associated with ancestral conditions in which fathers remained within nesting groups.

A common factor of eusocial species with ancestral bi-parental care and extended alloparental male worker castes, is that nests tend to be concentrated around limited resources, such as food or shelter. Nesting around limited resources may promote philopatry and facilitate communication between parents; breeding males will have assured parentage, and kin selection associated with evolution of extended alloparental care in the male is achievable (Thorne, 1997; Trumbo, 2012; Suzuki, 2013). Ancestral monogamy and nest building in the Hymenoptera is a primary factor contributing to the evolution of eusociality in the order (Andersson, 1984; Boomsma, 2007; Hughes et al., 2008; Boomsma, 2009). It would be expected that with nest building, resident helping fathers would be selected for, as relatedness and helping investment in offspring is assured (Queller, 1997). Contrary to potential advantages for resident fathers, paternal care is not widespread in the Hymenoptera. A potential case of helping fathers, but directed towards their mates, is in male Thynninae wasps that feed and carry wingless female partners. By carrying females to food sources, Thynninae males provide nuptial gifts to partners, which increases the fitness of the partner and hence offspring (Thornhill, 1976; Alcock, 1981). Beyond these helping partners, paternal care is mostly absent (though see 1.4.4. A note on the unusual case of male helping in the wasp *Trypoxylon politum*). A degree of paternal care is expected within confined nesting, but because hymenopterans (both present day and likely ancestral) must search the surrounding areas for food (females are life insurers – Queller & Strassmann, 1998), this allows the unguarded female mate opportunities for extra-pair copulation (Jaffe, 2008). In contrast, male helpers typically belong to eusocial societies defined as 'fortress defenders', in which groups stay within the close confines of a resource (Queller & Strassmann, 1998).

1.3.2. Absence of male hymenopteran helpers: morphological restrictions

A second explanation for the rarity of male helping behaviour in the Hymenoptera, is that males lack morphological traits which are present in the female sex that promote helping and ultimately altruism. The absence of morphological adaptations such as a stinger or pollen carrying structures may hinder the evolution of behaviours such as brood provisioning or nest defence (Starr, 1984a; Starr, 1985a). In the aculeate Hymenoptera, the female ovipositor has been modified into a stinger over the course of evolution, and hence this modification is not relevant to males (though males of some species do possess abdominal spikes much like a stinger – Starr 1984a; Starr 1984b). The stinger may have facilitated the evolution of nest building and eusociality in the Hymenoptera, given the advantages of communal nest defence (Lin & Michener, 1972). In hymenopteran in which the stinger is absent (e.g. the stinger has been secondarily lost), communal defence is maintained by evolved traits such as strong biting mandibles and formic acid sprays – and therefore the absence of a stinger does not fully limit males in theory (Cavill & Robertson, 1965; Andersson, 1984). However, a stinger does allow for proficient defence against large vertebrate nest predators (Starr, 1985a; Schmidt, 1990).

The apparent deterrent of the stinger may be highlighted in male *Polistes* paper wasps, which will often mimic stinging action with their aedeagus when held, albeit without possessing any actual means to harm the attacker or handling observer (Starr, 1984b; pers. obs.). Eusociality in the Hymenoptera has multiple lineages, and the degree of sexual dimorphism is narrower in some social species than others, resulting in similar morphological traits between the sexes (Boomsma et al., 2005; Hughes et al., 2008; Peters et al., 2017). Given that in species without extreme sexual dimorphism there is no defined male worker caste, lack of altruistically-linked morphological traits may hinder the evolution of helping but is unlikely to be a primary explanation for the rarity of it (Kukuk et al., 1989).

1.3.3. Absence of male hymenopteran helpers: relatedness asymmetries

The third explanation for the lack of hymenopteran male helping is attributed to the haplodiploidy sex determination system, in which asymmetries in relatedness exist between the sexes. In eusocial species possessing nonbreeding male helpers, both sexes are often diploids. Diploid male helpers are found in the described eusocial species of mole-rats *H. glaber* and *F. damarensis*, *Synalpheus spp.*, and termites (Jarvis, 1981; Duffy, 1996; Thorne, 1997).

Hymenopteran males face two evolutionary strategies, of either staying on the natal nest and raising siblings or dispersing to mate. Ostensibly, fitness derived from relationship values appears equal for staying to help verse dispersing to mate. Using a simple model of a hymenopteran species with a single singly mated queen, nesting with her offspring; male offspring will be related to their brothers and sisters by $r = 0.5$ (life-for-life relatedness), but also to their partners' offspring by $r = 0.5$. Though males pass on all their genes to daughters ($r = 1.0$), they never contribute to the genetic material of the partners' male offspring ($r = 0.0$), which are produced from unfertilised eggs. Therefore, as the partners' male offspring dilute the high relatedness gained from daughters, males are equally related to their nesting partners offspring as to siblings on the natal nest (given that sex ratios and nest productivity is also equal). However, males may exploit high relatedness with daughters by biasing investment towards them rather than the partners' male offspring (if investments are limited, e.g. energetic and time costs). Most hymenopteran males cannot bias helping towards daughters on the partner's nest, but they have evolved traits that favour copulatory success, increasing daughter production (such as multiple mating, receptive-female detection, and mating plugs) (Hamilton, 1972; Boomsma, 2007). Therefore, males ultimately disperse from the natal nest to mate, with evolution favouring traits that increase copulation success and daughter production ($r = 1.0$) - over traits that may benefit helping (i.e. $r = 0.5$ to siblings and the partners' combined female and male offspring).

Observations of *Trypoxylon spp.*, in which males do sometimes remain on the breeding nest to guard mates and protect brood, reveal that the female appears to lay eggs of different sexes in random order on the nest with no apparent visual differences between the sexes of brood (for example, enlarged cells) (Hamilton, 1972; Brockmann & Grafen, 1989). Hamilton (1972) suggested that because in other solitary Hymenoptera the eggs of different sexes are laid sequentially with no randomisation, the males' primary source of fitness in *Trypoxylon* is therefore to mate with the partner, and the male is tricked into defending unrelated male offspring as the breeding male cannot bias helping towards daughters (though see 1.4.4. A note on the unusual case of male helping in the wasp *Trypoxylon politum*). The lack of male ability to skew sex ratios on the nest likely stems from them often being short-lived, with lack of within-nest kin recognition, and in not having the same advantage as the mother/female partner in deciding primary skew (i.e. by laying eggs) (Hamilton, 1972; Trivers & Hare, 1976; Kümmerli & Keller, 2009; de Souza et al., 2017). This further supports the idea that the ultimate strategy of males is to mate and produce highly related daughters.

Evolutionary selection on males to disperse and mate verses staying and helping contrasts with that of females. Females should bias investment towards full-sisters (r = 0.75), but under most circumstances they cannot influence the equal sex ratio of the egglayer (split sex ratios likely evolved in some species after fixed caste commitment, Gardner et al., 2011). Therefore, the decision to depart and start a new nest or stay and help is reliant on the potential output (in terms of productivity) of raising offspring (r = 0.5) or siblings (r = 0.5) (Strassman & Queller, 2007). Males should bias investment towards daughters (r = 1.0), but like females cannot influence the equal sex ratio of the egglayer (Hamilton, 1972). However, in evolving traits that increase copulation success, males can focus on increased daughter productivity (which per capita is skewed fitness-wise, and most likely always more advantageous than helping siblings, as r = 1.0 to daughters is twice that of r = 0.5 to siblings).

Male selection to depart the natal nest to mate may also feedback into the evolution of female cooperation. In most Hymenoptera, male-biased dispersal and female philopatry is common (Johnstone et al., 2012). Male-biased dispersal may weaken the effect of diluted relatedness when individuals establish new nests, as these groups will be composed of highly related sisters, and hence a subset of sisters giving up reproduction to gain indirect fitness by helping is selected for (Johnstone et al., 2012; however, direct fitness benefits may drive grouping in some species with low relatedness, see Leadbeater et al., 2011; Field & Leadbeater, 2016). Therefore, haploid males may attribute to why worker evolution is at least female-biased.

1.3.4. Absence of male hymenopteran helpers: protected invasion hypothesis

The final main explanation for the rarity of male helpers also relates to haplodiploidy, in that dominant maternal supporting alleles are likely to be more resistant to genetic drift than dominant paternal supporting alleles (Reeve, 1993). The protected invasion hypothesis (Reeve, 1993) argues that female sex-biased helping in eusocial species with haplodiploidy relates to the nature of hymenopteran males being haploid and females being diploids. In a finite population, the probability of a new phenotype being maintained over time will rely on the magnitude of its selection. If rare evolutionary innovations, such as mutant alleles supporting parental or alloparental helping behaviour are expressed via heterozygosity, then active positive selection will be limited to the diploid sex (Reeve & Shellman-Reeve, 1997). Protected invasion may explain why multiple linages of eusociality have arisen in the Hymenoptera in comparison to other orders (Hughes et al., 2008), as altruistic alleles would have a larger magnitude of effect in the female sex; and why in other species where both sexes possess diploidy, both male and female helpers have evolved (Reeve, 1993). However, the protected invasion hypothesis does require that mutant alleles supporting helping have overdominance (Gardner et al., 2011).

1.3.5. Absence of male hymenopteran helpers: other explanations

Beyond these four main explanations, some further aspects of haploidy may influence male helping. First, haploid males produce colonial sperm, whilst males of diploid species produce genetically diverse sperm. In diploid males, there might be an advantage in remaining and continuously mating with a female (such as in termite kings), as the fittest sperm combinations are more likely to be produced and inseminate the female. In contrast, there is no pressure for sperm selection in haploid males, as every sperm is genetically identical (Jaffe, 2008). Second, contrary to resulting in a lack of male helping, haplodiploidy with inbreeding may promote male helping as males become more related to offspring (Davies & Gardner, 2014). However, Davies & Gardner (2014) point out that inbreeding has highly detrimental effects in the Hymenoptera, and may result in homozygous diploid males which are often either sterile or have subpar diploid sperm (that may further result in sterile triploid female offspring if males manage to mate) (van Wilgenburg et al., 2006). Avoidance of inbreeding may therefore be a stronger selection pressure than the potential to help. In hymenopteran species that do possess high levels of inbreeding (such as if males mate within-nests or societies possess polyphenic ergatoid wingless males), no stark observations of male helping have been reported. In these inbred Hymenoptera, species typically have traits to avoid detrimental inbreeding effects, which may also negate male helping, such as colonial queens, possible multilocus complementary sex determination, male-biased dispersal, and asynchronous production of the sexes (Keller & Passera, 1993; Cook & Crozier, 1995; Thurin & Aron, 2009;

Pearcy et al., 2011; Klein et al., 2016; Heinze, 2017). This contrasts with other eusocial haplodiploid species, such as the simple eusocial Thysanoptera gall thrips, that do possess male helpers in the form of a solider caste which are highly related and inbred (Crespi, 1992; Chapman & Crespi, 1998; Chapman et al., 2000). Indeed, inbreeding is also found to a degree in eusocial diploid species with male helpers, such as the mole-rat *H. glaber* and in some termites (Reeve et al., 1990; Chapman et al., 2000). Why inbreeding facilities male helping in these species and not in the Hymenoptera is not fully understood, but in the gall thrips it might be associated with local mate competition, dimorphic similarities between the sexes, and 'fortress defence' (Queller & Strassmann, 1998; Chapman et al., 2000). It could then be hypothesised that hymenopteran males which do remain in the nest and compete for mates, such as ergatoid male ants, have evolved nesting-related traits after female helpers have been evolutionary established – and hence males are excluded from becoming helpers. Life history may also contribute to lack of male helping, such if (as mentioned, as a trait to avoid inbreeding) most males are asynchronous produced at the end of the nesting cycle and have limited opportunities to help raise brood (Wilson, 1971; Judd, 2000). Another explanation is if haploid males are more susceptible to disease, and hence at a disadvantage in social groups compared to diploid females (O'Donnell & Beshers, 2004). Finally, if males not mating results in higher risks in the evolution of alloparental care, because there is no secondary fitness alternative males will be at a loss; whilst unmated females are able to lay unfertilised male eggs as a backup to alloparental care. In diploid species, this risk would be equal between the sexes (Rautiala et al., 2014).

In summary, there are various potential reasons accounting for the lack of widespread male helping in the Hymenoptera, and explanations likely all interplay to various degrees. What is lacking beyond theories of why males do not help, is actual empirical evidence of what behaviours males do perform and specifically data on them *not* helping. It has widely been presumed that males do not help, but low levels of helping have been reported in some species (see 1.4. Paradoxical acts of helping in natal male Hymenoptera). This thesis attempts to bridge the gap between theories of why there is a lack of a strong male worker caste, and how males can contribute to the nesting group beyond being reproductive extensions.

1.4. Paradoxical acts of helping in natal male Hymenoptera

Most social hymenopteran males spend a period of their life on natal nests before eventually dispersing, and these young males appear to avoid being forced from the nest by resident females (Hamilton, 1964; Hamilton, 1972). One possibility of why males are not forced from the nest by females is if they are of a relatively low cost (resource-wise) to raise (Trivers & Hare, 1976; Aoki & Moody, 1981; Bourke, 2015). If males receive increased fitness from taking a small amount of nest resources, then less related but low-cost brothers may increase the indirect fitness of sisters. Few studies have tested whether males do indeed contribute or not to the nest beyond a sperm delivery service. There are anecdotal (and few tested) reports across the Hymenoptera of natal males performing supposed helping behaviours (reviewed in Table 1.1). Observations of helping-like behaviour in males can broadly classified into three categories that relate to natal nesting: food distribution to brood; nest defence; and nest thermoregulation.

1.4.1. Helping in natal hymenopteran males: food distribution behaviour

Sharing food may facilitate long-lasting relationships between individuals, and contribute to the establishment of social groups (de Waal, 1989). Voluntary exchange of food between two individuals is attributed to social relationships that are either reciprocal and/or beneficial to kin (Hamilton, 1964; Trivers, 1971). Species such as vampire bats *Desmodus rotundus* demonstrate reciprocal food sharing, whereby individuals will give up food if the recipient will likely return the favour to the actor in the future (Wilkinson, 1990; Carter & Wilkinson, 2013; Carter & Wilkinson, 2015). One-way food sharing may also be beneficial if it is with close kin, increasing the indirect fitness of the actor (Hamilton, 1964).

The social Hymenoptera are regarded as having a 'common stomach'. The ability to store, regurgitate (if ingested), and transfer liquid food or water to nestmates is amplified by the effect of many individuals on the nest contributing and drawing from the same network. The common stomach allows not only sharing of food, but information transfer between nestmates of the groups' resource needs (Karsai & Wenzel, 2000; Karsai & Schmickl, 2011; Schmickl & Karsai, 2016). Individual interactions within the food sharing network can be defined as food distribution, whether between many nestmates or a solitary parent provisioning brood. Most hymenopteran females will carry out food distribution in their lifetime, from queens caring for the first brood that will become the first emerged workers, to foraging workers returning to the nest with food, or young individuals receiving food from foragers and helping with its distribution (Hunt, 2007).

Table 1.1. Occurrences of helping behaviour in natal adult males of social Hymenoptera (underlined species *Polistes lanio*, used in this thesis / blank spaces indicate unknown function).

Family	Species	Description of Behaviour	Link to Age / Sexual Immaturity	Association to Nest	Putative Function of Behaviour			References
					Food Distribution	Nest Guarding	Nest Thermoregulation	
Apidae	*Apis mellifera*	Contribution to nest temperature through general thermogenesis.		Natal			Yes	Harrison, 1987
	Bombus affinis; B. bimaculatus; B. bimaculatus; B. griseocollis; B. pennsylvanicus	Incubating pupae.	Most prominent during first 24–36 hours after emergence (pers. obs. in Cameron, 1985).	Natal			Yes	Cameron, 1985
Crabronidae	*Microstigmus nigrophthalmus*	Darting at small heterospecific insect intruders.		Related to Nestmates		Yes		Lucas et al., 2011; Lucas & Field, 2011
Formicidae	*Camponotus herculeanus; C. ligniperda*	Receiving and distributing liquid food to emerging and newly emerged nestmates.	Most prominent during phase of sexual immaturity.	Natal	Yes			Hölldobler, 1966
	Formica polyctena		Rarer in *F. polyctena* than *Camponotus spp.*, has shorter phase of sexual immaturity.	Natal	Yes			
	Cardiocondyla sp.	Ergatoid w ingless males carrying brood, explanation unknown?		Presumed Natal				Santschi, 1907 (in Hamilton, 1972)
Halictidae	*Lasioglossum erythrurum; L. sp.*	Nest entrance guarding by a distinct male polymorph.	Sexually mature.			Likely inter-sex competition rather than nest guarding		Rayment, 1955; Houston, 1970 (but likely inter-sex competition, see Danforth & Desjardins, 1999)
Vespidae	*Belonogaster spp.*	Larval provisioning and nest maintenance.			Yes			Pardi, 1977 (in Lucas & Field, 2011)
	Mischocyttarus drewseni; M. flavitarsus; M. labiatus; M. mastigophorus	Receiving, mastication, and distribution of solid food to larvae. Wing fanning in *M. mastigophorus*.			Yes		Species Specific	Jeanne, 1972; (M. Litte, per. coms. in Hunt & Noonan, 1979); O'Donnell, 1999 (note, males make unusual aggressive interactions with females nestmates for food in *M. mastigophorus)*
	Polistes biglumis; P. fuscatus; P. ferreri; P. major; P. instabilis; P. exclamans; P. gallicus; P. metricus; P. riparius; P. snelleni; P. versicolor; P. jokahamae; <u>P. lanio</u>	Receiving, mastication, and distribution of solid and liquid food to larvae; Wing fanning in *P. biglumis, P. fuscatus, P. ferreri, P. major, P. instabilis, and <u>P. lanio</u>*; Foraging and distributing nectar in *P. instabilis;* Giving alarm in *<u>P. lanio</u>.*	Males aged zero to 31 days old in know n species (till departure).	Natal or Presumed Natal	Yes	Species Specific	Species Specific	Weyrauch, 1928 (in Hunt & Noonan, 1979);Steiner, 1932 (in West-Eberhard, 1969); Heldmann 1936 (in Hunt & Noonan, 1979); West-Eberhard, 1969; Hunt & Noonan, 1979; Makino, 1983 (in Makino, 1993); Cameron, 1986; Valadão, 1986 (in Sinzato et al., 2003); Makino, 1993; O'Donnell, 1995; Souza 1998 (in Sinzato et al., 2003); Sinzato et al., 2003; Giannotti, 2004
	Ropalidia cyathiformis; R. marginata; R. plebeiana	Receiving, mastication, and distribution of solid food to larvae. Wing fanning in *R. cyathiformis* and *R. marginata*	In *R. marginata* males aged zero to four–eight days old (till departure). In *R. cyathiformis* males reside on the nest, but distribution of food to larvae behaviour is rarer (mean 19 days on nest).	Natal or Presumed Natal	Yes		Species Specific	Gadagkar & Joshi, 1984; Kojima, 1993; Sen & Gadagkar, 2006, 2011
	Vespa orientalis	Receiving, mastication, and distribution of solid food to larvae.			Yes			Occurs infrequently (J. Isnay, per. coms. in Hunt & Noonan, 1979)
	Vespula flaviceps lewisii?; Vespula spp.	Receiving, mastication, and distribution of solid food to larvae.			Yes			Shida, 1952 (in Yamane, 1969); Sometimes uncommonly and in abnormal circumstances in other *Vespula spp* (R.D. Akre, per. coms. in Hunt & Noonan, 1979)

The act of food distribution is less prevalent in hymenopteran males than females. However, there have been reports of adult males giving up food voluntarily to both adult and brood nestmates (Hölldobler, 1966; Hunt & Noonan, 1979; Cameron, 1986; Giannotti, 2004; Sen & Gadagkar, 2006). Most recorded examples of male food distribution can be found among the social wasps, particularly in newly emerged natal males of eusocial *Polistes* paper wasps. In *Polistes*, female foragers bring back large balls of insect prey to the nest, such as Lepidopteran caterpillars. On returning to a nest with forage, female foragers may give up the food to other adult nestmates, including natal males, who will then masticate the food mass before distributing it to brood (Cameron, 1986). Males *Polistes* never forage prey directly, and only rarely have been observed foraging and provisioning larvae with water and nectar (in *Polistes instabilis* – O'Donnell, 1995). Taking food from females on the nest, and subsequent food handling and distribution, appears to be the limit to male provisioning activity. Compared to females, males are normally slower in handling food and feed fewer brood per solid forage load (Cameron, 1986). Sen & Gadagkar (2006) recorded in the simple eusocial wasp *Ropalidia marginata* that males took approximately three times as long to masticate food balls compared to females, and only fed a single larva per load whilst females fed two. Whilst males (particularly in the wasps) do help with food distribution, which may increase the fitness of the recipient and the indirect fitness of the distributing male if related, males may be considered inferior helpers that eventually depart the nest to mate in comparison to phenotypically committed female worker caste.

1.4.2. Helping in natal hymenopteran males: nest defence behaviour

An evolutionary arms race exists between prey and host against predators, parasitoids, and pathogens (and to an extent harmful conspecifics). A wide array of mechanisms to counter biotic threats have evolved, from reducing individual risk by clustering in large aggregations, developing immune system responses, directly avoiding capture and detection, to actively fighting off attackers and intruders (Barnard, 2004). Fighting back is a risky strategy, as individuals put themselves within harms reach, and it may be resorted to as a last line of defence (Ratner, 1975). Individuals may not only fight back to defend themselves, but also come to the defence of others in which they have mutualistic (whether reciprocal, manipulative, or by-product) and kin-based relationships (Hamilton, 1964; Hamilton, 1971; Ostreiher, 2003; Clutton-Brock, 2009). Kin defence is a strong selective pressure, particularly if high levels of investment have been made into survival and future fitness of dependent young (Pianka, 1970; Stearns, 1976). For example, long-lived baleen whale Mysticeti mothers (and groups) physically ward off attacks on calves from predatory orcas *Orcinus orca* attacks, defending dependent kin (Ford & Reeves, 2008). It is then no surprise that eusocial insects actively defend their nests from predation, as the nest offers a grand prize of large amounts of biomass for predators (Wilson, 1975)

The insects have evolved a diversity of traits to actively fight back against threats (Schmidt, 1990). To counter attacking threats to their nests, eusocial insect societies have evolved novel traits in defence of their kin beyond simple biting and grabbling at intruders, such as suicidal rupturing (by autothysis or dehiscence) of defenders in termites and ants, *Skatitermes* termites that precision defecate on intruders, ants species which ooze or spray formic acid, and the evolutionary modification of the ovipositor into a stinger across the Hymenoptera (Cavill & Robertson, 1965; Hermann & Blum, 1981; Prestwich, 1984; Starr, 1984a; Starr, 1985a; McCorquodale, 1989; Schmidt, 1990; Weiss, 2006; Shorter & Rueppell, 2012). In the social Hymenoptera, communal defence of the nest has likely been critical in species that have passed the evolutionary transition towards sociality (and eusociality); whether by actively attacking intruders (e.g. in large groups, with enclosed defendable nests), or passive defence by camouflaging and building nests in inaccessible places to predators, with the potential release of chemical deterrents (e.g. in small groups, in which members must leave the nest unattended) (Jeanne, 1975; Post & Jeanne, 1981; Andersson, 1984; Smith et al., 2001; Starr, 1984a; Starr, 1985a; Dani et al., 1996; London & Jeanne, 2000). Not only does group living allow communal defence, but if nests are destroyed after conflict, then those living in large groups may rebuild nests faster than smaller groups (Strassmann et al., 1988; Makino, 1989). Within female castes of eusocial Hymenoptera, some members may be more specialised to defend the nest, such as if caste size dimorphism lends some workers to be better defenders than others (e.g. larger 'major' castes in ants – Hölldobler & Wilson, 1990). Further, the division of labour in nest defence among females likely correlates with investment and reproductive state between queens and workers. In the simple eusocial *Polistes* paper wasps, nest defence is performed frequently by the resident reproductive queen, who has invested heavily into survival of the brood; whereas potential reproductive workers and gynes may avoid defence, if they have alternative fitness options (such as abandoning the nest and laying their own eggs in a new nest) (Fishwild & Gamboa, 1992; Judd, 2000). Alternatively, in complex eusocial species which may have a specialised single reproductive, queen loss in risky defence would result in the collapse of the nesting group, and sterile workers left at a fitness dead-end; and so workers intensively defend the nest, with the evolution of failsafe traits such as nest fissioning, multiple queens, emergency queen cell production, and worker male-reproduction (Fell & Morse, 1984; Nonacs, 1988; O'Donnell, 1998; Henshaw et al., 2004; Kronauer, 2009; Ratnieks & Helanterä, 2009). Given that the males' optimal fitness is achieved through mating, selection to stay and actively defend the nest at the potential cost of its own life is likely to be weak (Judd, 2000).

Despite the risks involved in active nest defence against attackers, males do perform nest defence to a degree in some species. Natal males may sometimes perform nest defence in two possible ways, by lunging and biting at smaller intruders and bluffing at larger threats; for example, in *Polistes* species males raise their wings in a similar threat posture as performed

by females, bluffing as they possess no stinger for attacking back with (Starr, 1984b; Giannotti, 2004; Figure 1.1). Small intruders, such as parasitoids, are mainly a threat to the brood and not the adult wasps directly, hence the only cost to male defence may be in energy and time expenditure (Brockmann & Grafen, 1989; Lucas & Field, 2011). Whether this defence is primarily for the benefit of the male or the brood is debatable. Lucas & Field (2011) report that males of Crabronidae social wasp *Microstigmus nigrophthalmus* actively defend nests from brood parasitoids, but they also remain on nests even once nests are devoid of brood; therefore, males may benefit from having these nests as safe perches in which to search for mates from in the area. Keeping the nest protected from intruders could personally benefit the defending males, and helping brood may be a secondary benefit.

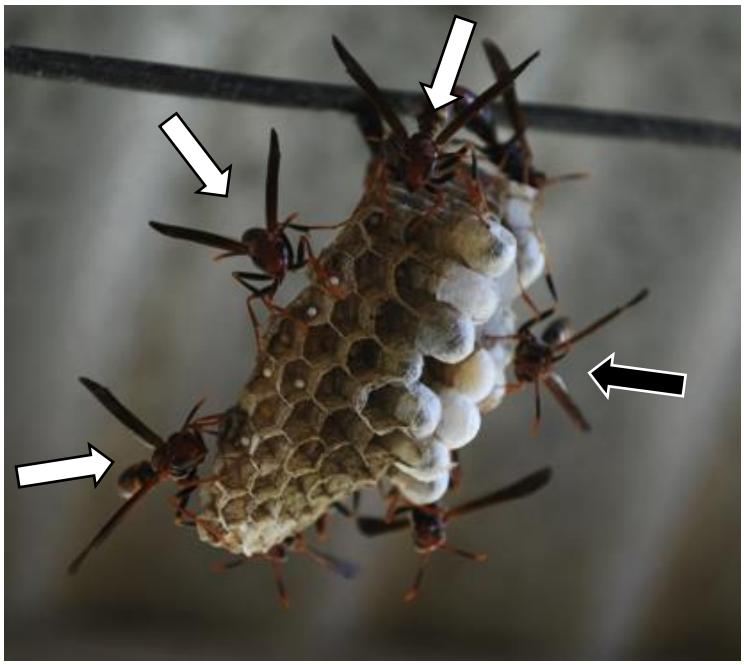


Figure 1.1. Male defensive bluffing in *Polistes versicolor* - males (black arrow) adopt the same threat posture of wings held in a V shape as female nestmates (white arrows), however, males do not possess a stinger in which to attack large threats.

1.4.3. Helping in natal hymenopteran males: nest thermoregulation behaviour

Climatic conditions have a large impact on offspring survival and development, and the ability to manipulate temperature (i.e. thermoregulate) to benefit developing offspring has evolved multiple times across the animal kingdom. Some of the best examples of animals establishing optimal temperature ranges for offspring are found in endothermic birds, in which parents directly transfer their own body heat to eggs via incubation (Leon, 1986).

Thermoregulation is vital in the insects, due to individuals having relatively small body masses even brief changes in the external environmental temperature can result in fast internal temperature changes (Heinrich, 1993). Most Hymenoptera species possess a degree of homeothermy, by which individuals can manipulate internal body temperature, but also change the temperature of the immediate micro-climate (Gibo et al., 1974; Willmer et al., 2005). Homeothermy in the Hymenoptera is essential for spatial-fixed nesting, as brood are immobile and have no self-regulating mechanisms to control body temperature, therefore relying on adult nestmates to provide optimal temperature ranges for development (Gardner et al., 2006). Maintaining this optimal temperature range for brood, results in increased brood growth, decreases in developmental abnormalities, and may influence post-emergence adult behaviour of the brood later in life – such as in task performance or caste-determination (Heinrich, 1993; O'Donnell & Foster, 2001; Johnson, 2002; Tautz et al., 2003; Stabentheiner et al., 2010). Adult nestmates may cool the nest by fanning wings to control airflow, or by collection of water droplets that are placed around nesting structures (Weidenmüller, 2004; Engels et al., 1995). Increasing heat within the nest is achieved by thermogenesis of adults, in contracting and relaxing flight muscles, either heating a general area of the nest or directly incubating brood structures (Heinrich, 1972; Bujok et al., 2002; Basile et al., 2008).

Most hymenopteran females will perform nest thermoregulatory activities in their lifetime, and this helping behaviour is also found in males. Males increase heat on the nest through metabolic heat creation, either by their presence (*A. mellifera*, Harrison, 1987; Kovac et al., 2009), or directly via incubating brood (*Bombus spp.*, Cameron, 1985). Male behaviours that cool the nest are seen in the activity of wing fanning (Cameron, 1986). However, it is difficult to explain the primary reason for nest thermoregulation in males. Male nest thermoregulation could be the result of direct helping effort, or a by-product of more selfish actions, such as exercising flight muscles or maintaining body temperature for self-survival and increased sperm viability (Heinrich, 1974; Stürup et al., 2013). Male thermoregulation could be altruistic, but also mutually beneficial if adult males have the same temperature demands as brood (i.e. an optimal temperature range for adult males is not of detriment to brood) – then both brood and adult males may increase fitness simply by association.

1.4.4. A note on the unusual case of male helping in the wasp *Trypoxylon politum*

The pipe-organ mud-daubing wasp *T. politum* is highlighted as a peculiar case of a paternal care, as it demonstrates conditions in which common male helping is feasible in the Hymenoptera, which is not realised in other species. Males of *T. politum* will guard a nest by chasing away male conspecifics and smaller heterospecific intruders. Nest defence against intruders in *T. politum* has likely evolved as a secondary function of mate guarding; though males will also sometimes perform 'spider-packing behaviour' (pushing prey items inside of the brood cells) and enlarging the resident mud nests by use of mandibular vibrations (Brockmann & Grafen, 1989). Similar mate and nest guarding behaviours are observed across sphecid (*Trypoxylon spp.* and *Pison strandi*) and Crabronidae (*Oxybelus spp.*) wasps (species with no extended alloparental care in either sex) (Peckham, 1977; Tsuneki, 1970; Brockmann, 1980; Hook & Matthews, 1980; Starr, 1984a). Because the female optimal sex ratio will be equal for male and female offspring, whilst males only pass on their genes through daughters and not their partners sons, then these male defenders are at a disadvantage because the female sex ultimately decides the primary sex ratio as she lays the eggs (Trivers & Hare, 1976). Eggs in *T. politum* nests are laid in random order, with no apparent visual differences between the sexes of brood, and hence a male cannot be selective in his individual helping effort to brood (Hamilton, 1972; Brockmann & Grafen, 1989). However, male *T. politum* may influence the egg laying decision of partners by providing a defence service. Described by Brockmann & Grafen (1989), male offspring are typically smaller than female, and hence need less food than females: in nests without a male guard, smaller male-biased offspring may be produced as the mother diverts less time foraging and more time defending the nest; in nests with a male guard, larger female-biased offspring may be produced as the mother spends more time foraging and less time defending the nest. Through the feedback mechanism of a nest receiving more female foraging activity, then the male defender may know that he is guarding a nest mainly containing his daughters. The advantage to the mother laying biasing sex ratio towards female production is the increased nest defence offered by the male. However, a male will equally guard brood cells containing male offspring as well as female in female-biased nests, and hence males are cheated out of fitness regardless. Brockmann & Grafen (1989) hypothesize that the benefit of mate defence outweighs that of nest defence in males, which provides a secondary source of fitness. This example of common male helping and influence of sex ratios in *T. politum* may be limited to this and similar species, due to the nature of mating and reproduction nests are built and provisioned with food before an egg is laid and sealed inside. Male helping may be supported by guaranteed parentage through mate guarding against conspecifics, low cost of defence against heterospecifics (defence against other species was directed towards parasites and predators, much smaller and weaker than the male), guarding from the safety of the nest may providing protection against larger conspecific predators (males never build their own nests), and importantly the ability to be able to judge

whether brood will be female-biased containing their shared genes or not (Brockmann & Grafen, 1989). In other eusocial hymenopteran species with mate guarding males that do remain on or in proximity to the nest, such as males in ergatoid ants and males of the wasp species *Polistes gigas* and *Synagris cornuta*, it is unknown whether mate guarding has been extended to common nest defence against heterospecifics (Foitzik et al., 2002; Longair, 2004; Lee & Starr, 2007). Though helping acts by these mentioned species may be unlikely, given that they possess ornate fighting appendages such as overly enlarged mandibles, which may limit their behaviour repertoires.

1.4.5. The adaptive value of hymenopteran male helping

Male helping is expected to be rare in the social Hymenoptera. Newly emerged adult males in some species rarely contribute to nest tasks, and are considered a detrimental drain (to the disadvantage of female members) on group resources until voluntary dispersal or forced departure by female nestmates (Trivers & Hare, 1976; Brian, 1983). As females will be selected to try and channel resources to more closely related sisters, female worker behaviour has likely evolved to counter the effects of undesired brothers on the nest. For example, 'cell-stuffing' in the paper wasp *Polistes dominula*, whereby adult males have their heads forced into cells whilst food is distributed to possible sister-brood around the nest (Starks & Poe, 1997). However, our review (Table 1.1) of natal male helping demonstrates that it is present (to a degree) in many other species. Helping in natal males can be described as thus: that it is often overlapping but inferior in performance compared to worker females; that it is likely of low cost, and therefore not in the same classification of the altruistic acts of worker females; and that it may be either weakly altruistic (low cost to the male, benefitable to the recipient) or mutually/by-product beneficial (benefit to both the male, and the recipient). However, most observations of male helping vary from personal communications in the literature, to claimed reporting of rare and abnormal behaviour. Given the numerous independent origins of eusociality in the Hymenoptera where male helping is reported, it could be concluded that male helping is more than just a freak occurrence, but we currently lack empirical studies to test this.

The under-appreciation of the potential importance of male helping is likely due to males being often thought as inconsequential to hymenopteran sociality, because male helping is not on the same level as that of the altruistic female workers (i.e. not as frequent or effective). Perhaps this has arisen because male helping behaviour often overlaps with that of the female, such that both males and the altruistic female workers will perform food distribution, nest defence, and thermoregulation. However, the evolutionary selection pressures on female and male actors may vary. To be able to further understand why and how males might help, we

must understand the conditions in which male helping can occur, such as: Do males delay dispersal after emergence from the natal nest, and if so what regulates eventual departure? Is there a kin-benefit to male helping, i.e. does helping increase male inclusive fitness? Is male helping rare and reliant on chance abnormal occurrences, or is it linked to a regular part of male life history?

1.5. Are helping males delayed dispersers?

Male-biased natal dispersal is common in the social Hymenoptera. In simple eusocial species, newly emerged adult females have the choice to either remain on the natal nest (or group if swarm founding) and help, or disperse to found new nests. Therefore, females have a variety of options in maximising inclusive fitness, to either gain indirect fitness on the natal nest by helping siblings; gain direct fitness by starting new nests; or inherit a dominant reproductive position and gain direct fitness on either the natal or new nests (West-Eberhard, 1982; Hunt, 2007; Leadbeater et al., 2011; Seppä et al., 2012; Field & Leadbeater, 2016 – some species can also drift between non-natal nests, gaining indirect fitness, see Sumner et al., 2007). In the complex eusocial species, female dispersal is limited by the social structure of the group and phenotype of the individual, such that female workers typically stay with the natal group whilst gynes (individuals destined to become new queens) depart to found new nests (Sundström, 1995; Peeters & Molet, 2009; Hamidi et al., 2017). Even more restrictive are male options, except for wingless ergatoid ant males: most males are either forced to or voluntarily depart from the natal nest. For example: female workers of desert leaf-cutter ants *Acromyrmex versicolor* forcefully carry males a distance from the nest before releasing them, not allowing them to return (Gamboa, 1975); conversely, in the honeybee *A. mellifera* males have the choice to disperse and then return to the natal group after unsuccessful mating flights to disperse again on later days (Harano, 2013); in swarm founding species, males show variation in ability to follow an emigrating swarm, in the wasps *Parachartergus fraternus* males do not disperse with the group, whilst in *Apoica pallens* males can (Howard et al., 2002; Mateus, 2011). Male-biased dispersal severely limits the potential for males to evolve helping behaviour, as philopatry is strongly linked to the evolution of sociality (Johnstone et al., 2012). Pressures on males to depart include the benefit to females in not wasting resources on lesser related brothers (given that females are usually stronger and more aggressive than brothers this choice is theirs), and to avoid highly detrimental inbreeding from haplodiploidy (Trivers & Hare, 1976; Brian, 1983; Polak, 1992; Pusey & Wolf, 1996; Starks & Poe, 1997; van Wilgenburg et al., 2006).

Despite male-biased dispersal in the social Hymenoptera, most dispersal is delayed in that males appear to spend a few days on the natal nest before departure (Hamilton, 1972).

Dispersal from the natal nest likely entails a high cost, both energetic and from risk of predation, and therefore if timing is not optimal then fitness will be reduced (Bonte et al, 2012). One possibility is that males delay dispersal until breeding opportunities or sites are available (Clobert et al., 2009). However, in the eusocial Hymenoptera a strong association is found between dispersal and reproductive maturation (Hamilton, 1972). Upon emergence as an adult, in many social (and some parasitoid) Hymenoptera, male maturation takes a few days, as sperm migrates from the testes to the seminal vesicles, with complementary swelling of the accessory glands; this defines a male as either immature or sexually mature (Hagen, 1953; Duchateau & Mariën, 1995; Quimio & Walter, 2000; Moreira et al., 2004; Moors et al., 2009; Araújo et al., 2010; Poidatz et al., 2017). Likewise, male dispersal and hence mating happens several days after adult emergence, this period of immaturity before mating being sometimes referred to as a 'obligatory pre-mating period' (Hagen, 1953; Gobbi, 1975 in Giannotti, 2004; Ruttner, 1976; Duchateau & Mariën, 1995; Tasei et al., 1998; Rhodes, 2002; Baer, 2003; Poidatz et al., 2017).

By remaining on the nest until reproductive maturity, males may benefit from optimal dispersal times related to mating availability/potential and reduced predation. They may also benefit from accelerated maturation and improved final mating condition, as they gain resources from the female worker caste (Litte, 1977; Hunt et al., 1982; O'Donnell & Jeanne, 1992a; Leatemia et al., 1995; Yuval et al., 2002; Costamagna & Landis, 2004; Hunt, 2007; Bonte et al., 2012). For the female, if the cost of sharing resources to brothers is low, then there will be selection to temporarily help these males, as female workers will still receive (albeit lower, $r = 0.25$) indirect fitness. For example, in the bumblebees *Bombus spp.* the cost of helping delayed dispersing males may be low, males do require large amounts of pollen after emergence but larger female gynes require more feeding and care during the larval stage (Baer, 2003).

An unexplored possibility is that whilst hymenopteran males are maturing on the natal nest they may invest, temporarily, in indirect fitness through helping. Hölldobler (1966) made a key observation, that natal male ants of *Camponotus herculeanus*, *Camponotus ligniperda*, and *Formica polyctena*, when receiving liquid food would sometimes distribute a portion to other nestmates instead of consuming it all selfishly. Hölldobler (1966) observed that *C. herculeanus* and *C. ligniperda* performed this helping behaviour for much longer periods than *F. polyctena* – the latter having a much shorter sexual immaturity phase. Despite this observation, the idea that male helping may correlate with sexual maturation has received little attention in the literature (Cameron, 1985). Helping on the natal nest by males could allow them to avoid female aggression (Bergmüller & Taborsky, 2005). Alternatively, a temporary male helping phenotype during this immature stage may maximise lifetime inclusive fitness.

If male helping is associated with maturation, then patterns of sexual maturity may create two distinct phases in a male's post-emergent life as an adult (Figure 1.2). First, when a male is sexually immature, it may remain on the natal nest and help, potentially gaining indirect fitness. Second, when a male is sexually mature, it may depart the nest to mate – gaining direct fitness. The final phase for a male should always be to disperse and mate, because the highest fitness payoffs arise for them through having daughters (relatedness, r = 1.0); therefore, helping during the proposed immature phase may represents a male 'making the best of the current situation' to maximise inclusive fitness (r = 0.5). Switches between phenotypes is also common in insects, particularly eusocial Hymenoptera. Within the worker caste, hormones may be associated with age polyethism between division of labour, such that workers will perform different tasks throughout their lifetime – and this is regulated by developmental hormonal changes (Robinson, 1987; O'Donnell & Jeanne, 1993; Giray et al., 2005; Shorter & Tibbetts, 2009). Additionally, between worker and reproductive castes, simple eusocial species possess plasticity between phenotypes with workers able to become reproductive queens (Sumner et al., 2006; Patalano et al., 2012).

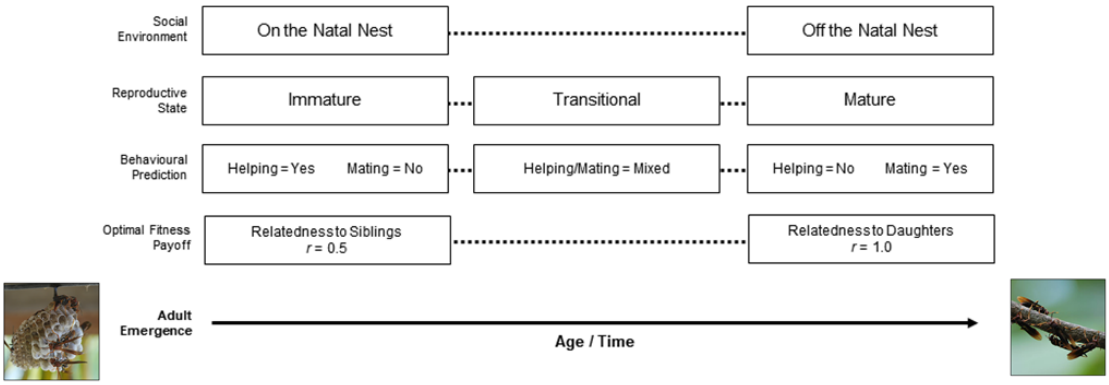


Figure 1.2. Hypothetical life history payoffs for hymenopteran males who help; optimal fitness payoff calculated as life-for-life relatedness (r = 0.5 to both sisters and brothers).

Developmental switches are likely to be hormonally regulated (Nijhout, 2002). In insects, the sesquiterpenoid juvenile hormones (JH) play a key role in a wide range of developmental and behavioural regulation, from reproduction, diapause, and polymorphisms (Riddiford & Truman, 1993; Wyatt & Davey, 1996; Goodman & Cusson, 2012). In the female Hymenoptera, JH (JH3) is associated with regulation of age polyethism (however, not in all species, see

Agrahari & Gadagkar, 2003), reproductive physiology, promotion of dominance behaviours, and dispersal flight activity (Barth et al., 1975; Röseler et al., 1984; Robinson, 1987; Robinson et al., 1991; O'Donnell & Jeanne, 1993; Giray et al., 2005; Bloch et al., 2000; Hartfelder, 2000; Shorter & Tibbetts, 2009; Tibbetts & Izzo, 2009; Smith et al., 2013 – it should be noted in *A. mellifera*, JH has lost its reproductive and behavioural dominance associations). The effects of JH in male Hymenoptera are less well understood. From what is known in two Apidae bee species, *A. mellifera* and the solitary carpenter bee *Xylocopa appendiculata*, JH in males appears to be a behavioural pacemaker. Applications of the JH analogue methoprene in males of these two species cause brain dopamine upregulation, dietary changes, and increased flight motor activity leading to earlier dispersal (Giray & Robinson, 1996; de Oliveira Tozetto et al., 1997; Harano et al., 2008; Sasaki et al., 2012; Sasaki & Nagao, 2013). Therefore, in the potential regulation of helping behaviour in males Hymenoptera, JH is a prime candidate.

An outstanding question in the understanding of helping by male hymenopterans is what regulates male dispersal, as the opportunity to help siblings is lost once a male departs the nest. Related to this is understanding sexual maturation on the nest, and the influence of hormones such as JH in regulation behaviour. A period of sexual immaturity sets the stage for male helping to potentially evolve, whilst developmental hormonal regulation of behaviour assures that helping is not an evolutionary dead-end, in that mature males do ultimately disperse to mate and produce highly related daughters (and do not end up helping on the natal nest).

1.6. Can males gain indirect fitness from helping?

Helping by natal males may be weakly altruistic or mutually beneficial. The ancestral breeding system of the social Hymenoptera is of monogamy (Hughes et al., 2008); thus, male helpers at the origin of sociality will be helping raise full-siblings. Typically, in Hymenoptera societies reproduction is monopolised by a single queen, and whilst she is present workers will not lay unfertilised male eggs (*Bombus spp.* – Paxton et al., 2001; *Polistes spp.* – Arévalo et al., 1998; Strassmann et al., 2003). Though, in some societies (including those of many complex eusocial species) production of males is shared by several females (to varying degrees, from sneak egglaying to significant production), with a subset of workers laying male eggs (*Polistes spp.* – Tsuchida et al., 2004; *Melipona spp.*, *Tetragona sp.*, *Scaptotrigona sp.*, *Plebeia spp.* – Tóth et al., 2002; *Dolichovespula spp.* – Foster et al., 2001; *Bombus spp.* – Owen & Plowright, 1982; Alaux et al., 2004; *A. mellifera* – Kirk Visscher, 1989; and many Formicidae – Choe, 1988). Having multiple maternities present reduces relatedness of natal males to each other. However, reduced relatedness of males to the brood may not affect expression of helping behaviour if

the action of helping is of low cost, as even a low level of indirect fitness can be enough to select for altruism (Strassmann & Queller, 2007).

To assess potential indirect fitness payoffs to males from helping (whether low or high), knowledge of relatedness among adult nestmates and brood is needed (both male and female). This requires examination of Hymenoptera mating and breeding systems, as the number of breeding females contributing to brood production will influence relatedness of natal males to the brood on the nest, and thus the potential for indirect fitness payoffs from male helping. Additionally, relatedness of adult females to the nest brood can influence male helping. Indirect benefits (and potential future direct benefits, Leadbeater et al., 2011) for female workers encourage acts of foraging behaviour to feed related brood. Male helping such as food provisioning requires that females perform initial foraging acts (to bring food to the nest), and therefore such behaviour is essentially cooperative between both sexes.

1.7. The evolution of male helping behaviour – social contracts and assured fitness

Females may allow males to stay if helping reduces the cost of males temporarily remaining on the natal nest. Males may therefore pay-to-stay: the male benefits from the reduced risk of not having to leave the nest whilst maturing, whilst female nestmates benefit from the extra help provided (Kokko et al., 2002; Bergmüller et al., 2007). However, pay-to-stay interactions are typically between unrelated individuals (Hamilton & Taborsky, 2005; Zöttl et al., 2013b). Given that natal males are likely related to nestmates pay-to-stay seems unlikely. Further, in species such as *P. dominula* where no helping has been observed, males were still present on the natal nest upon emergence for a time (Starks & Poe, 1997).

If male helping carries any indirect fitness benefits, then the fitness of the brood they help partially rear must be assured for the long period of development they have after the male has left the nest (Gadagkar, 1990; Queller & Strassmann, 1998). A consequence of hymenopteran males eventually departing the nest, might be that any helping is only temporary – males will never be on the nest long enough to raise brood from egg to adulthood. Therefore, assured fitness is uncertain. Males therefore must rely on their longer-lived resident sisters to rear brood they have partially invested in (Queller & Strassmann, 1998). This reliance on other nestmates to complete partial fitness investment has similarities to the idea that female Hymenoptera must be 'life insurers', in which they rely on nestmates to raise brood if they were to die (Gadagkar, 1990; Queller & Strassmann, 1998). For male fitness returns to be assured, then females must be long lived and related to the brood-offspring, so that females

are able and willing to help once the male has departed (Landi et al., 2003; Smith et al., 2003; Lucas & Field, 2011).

Further support for the idea that male helping is at least mutually beneficial, lies in its likely evolutionary pathways. In the wasp *T. politum*, where unusual paternal helping is present in the form of nest defence, helping behaviour as likely arisen as an extension of mate guarding behaviour (Brockmann & Grafen, 1989). It could be hypothesised that similarly, natal male food distribution and thermoregulation could have evolved as they were beneficial to the male, with leftover food or released heat benefiting related brood. To test this, we need to show that male helping is not a random occurrence, but that it is a distinct life history trait.

1.8. The *Polistes* paper wasps as a model system

The Polistinae paper wasps, specifically of the *Polistes* genus (deriving their name from the Greek for 'city-founder'), have emerged as an ideal model in the study of social evolution; in which females fight for dominance over reproduction and help on the nest to raise nestmate offspring (Pardi, 1948; Hunt, 2007; Jandt et al., 2014). *Polistes* is a simple eusocial wasp, with females possessing flexible reproductive castes throughout life, with no fixed commitment (West-Eberhard, 1969). *Polistes* is also the only (at least non-invasive) cosmopolitan genus of social wasp (Carpenter, 1996). Paper nests can be found in vegetation, as well as around human built structures (usually in places where they are left undisturbed – pers. obs.). Nests are typically open with no paper envelope, facilitating observation of behaviour on the nest such as adult interactions with brood. New nests are independently founded, being initiated by one or more females (Hunt, 2007). These new nests are classified as foundress associations, female nestmates may be sisters or of less and no relatedness (to the population average) (Leadbeater et al., 2011; Field & Leadbeater, 2016; see Chapter 2). Occasionally (but more frequently in the tropics), foundress associations are renesting groups, consisting of a mother and her daughters (Pickering, 1980). In terms of sociality, the nesting cycle starts as either semi-social (single generation of individuals) or eusocial (overlapping generations, mother with her daughters). Once the first generation of brood has been raised and the first natal individual emerges, the nests are classified as being 'post-emergence nests'. The post-emergence stage of the nesting cycle represents the highest peak in nest productivity, until the eventual decline and abandonment (West-Eberhard, 1969). Sociality in the post-emergence stage is eusocial, with overlapping generations and cooperative care of young. A nest typically consists of a single 'comb' (being an individual unit of cells joined together with a pedicel, see Figure 1.1), but occasionally some species build multiple combs within a relatively short distance that are simultaneously utilised (likely in response to parasitism – Jeanne, 1979). In temperate species, semi-social foundress associations are typically initiated in the spring, with

nest abandonment and subsequent diapause of mated females in autumn (West-Eberhard, 1969). Tropical species go through the same stages of the nesting cycle, except being perennial (with some variance in population number, with more nests in the wet compared to dry season) all stages of the nesting cycle exist simultaneously in the population (West-Eberhard, 1969; Pickering, 1980; O'Donnell & Joyce, 2001). Males are normally asynchronously produced (mainly by the queen at the end of the nesting cycle, but sometimes appear as 'early males' which may be sterile diploids (see 1.3.5. Absence of male hymenopteran helpers: other explanations) or fertile (they may be able to inseminate individuals that go on to be replacement queens in annual temperate species) (Pickering, 1980; Strassmann, 1981b; Arévalo et al., 1998; Tsuchida et al., 2004; Liebert et al., 2005; Hunt, 2007).

The mating behaviour of *Polistes* (and Vespidae in general) is relatively understudied. Mating in Vespidae usually takes place off-nest, pertaining to its difficulty in observation, and furthermore is disrupted by the artificial conditions of the laboratory (Kovacs et al., 2008). What is known from few natural observations is that *Polistes* males mainly possess two alternate mating strategies, of defending territories (or leks) (such as around bushes or overhanging twig perches) or patrolling (or roaming) along repeatedly used female flight paths (variance in these behaviours may be linked to male body size differences – Post & Jeanne, 1983; Beani & Turillazzi, 1988). Both males and females seem to possess sex pheromones that allow chemoanemotaxis-assisted mate location (Reed & Landolt, 1990). However, there is likely much diversity in mating behaviour within the cosmopolitan *Polistes* genus, for example males of *P. gigas* engage other males in ritualistic combat around nests (Lee & Starr, 2007), and *P. gallicus* males scramble compete in unisex swarms for females which land near male aggregations (Beani & Turillazzi, 1990). Most observations of successful copulations have been in temperate species, as observing off-nest mating in tropical species may be hindered by the dense vegetation found in these regions (for example *P. dominula* is commonly studied along relatively open field-margins in Spain, whilst tropical species in Panama and Trinidad can be found in abundance among abandoned buildings near or in forested zones – pers. obs.). Of the mating strategies employed by the tropical study species in this thesis (see below), territory defense is observed in addition to potentially rarer opportunistic mating on pre-emergence nests (West-Eberhard, 1969; Polak 1992, 1993 in *P. canadensis*; pers. obs. in *P. lanio*). It is likely that in alternate to territory defense, patrolling also exists. Occasionally natal males may attempt to copulate with other natal females (likely sisters/mothers), but are aggressively rejected by the female recipients (West-Eberhard, 1969 in *P. canadensis*; pers. obs. in *P. lanio*). Given the diversity of, and often anecdotal, sexual behaviour observed in males; there is need for further empirical studies in male Hymenoptera behaviour (Beani et al., 2014).

The *Polistes* paper wasps are excellent models for examining male hymenopteran helping behaviour. Here we study two tropical simple eusocial *Polistes* species found across the South American continent, being *Polistes canadensis* (Linnaeus) and *Polistes lanio* (Fabricius) (Carpenter, 1996). The reason for selecting this species is thus. First, *P. canadensis* and *P. lanio* are sister species, sharing similar evolutionary history, nesting habitats, and social structures; this facilitates experimental flexibility (i.e. in developing shared molecular markers), with cross-species comparisons (Santos et al., 2015). Second, male helping has been observed across a range of *Polistes* species, including *P. lanio* (underlined in Table 1.1) in which occurrences of all three categories of helping behaviour of food distribution, nest defence, and thermoregulation have been reported in natal males (Giannotti, 2004). Third, there is high potential for inclusive fitness benefits in helping kin; a previous study using microsatellites found high relatedness between female brood-nestmates in *P. canadensis* (Lengronne et al., 2012). Fourth, males are produced throughout the year in the tropics as the nesting cycle is perennial (Giannotti & Machado, 1994a). Lastly, despite *P. canadensis*' relatively prominent status in the literature (West-Eberhard, 1969), tropical species are generally understudied; much work is done on temperate species, but these species experience different selection pressures, such as sudden winter nest termination and diapause. The tropical aspect of *P. canadensis* and *P. lanio* is particularly important; *Polistes* likely originated in the tropics and so tropical species likely represent the same environmental conditions experienced by the first *Polistes* societies (Carpenter, 1996; Santos et al., 2015).

1.9. Aims of the thesis

The aim of the thesis is to investigate male behaviour in terms of the regulation of helping behaviour and potential indirect fitness gains. To achieve this aim, we study two sister species of Neotropical simple eusocial Polistinae paper wasps, *P. canadensis* (Chapters 2 and 4) and *P. lanio* (Chapters 3, 5, and 6).

1.9.1. Chapter 2 and 3: What is the genetic structure of tropical *Polistes* nests?

Kinship between group members may select for cooperative behaviours that increase indirect fitness, if the benefit of high relatedness between kin outweighs personal direct fitness (Hamilton, 1964). Here we analyse the genetic structure in nesting groups of two tropical *Polistes* species, to infer fitness benefits of both female and male nestmates (Chapter 2, *P. canadensis*; Chapter 3, *P. lanio*). The *Polistes* paper wasps are a cosmopolitan genus, but genetic structure data is almost entirely lacking in tropical species, and therefore assumptions of fitness payoffs for different strategies cannot be assumed from temperate *Polistes* alone. Tropical and temperate *Polistes* likely experience differing social and abiotic environmental pressures, in that tropical nesting cycles are not limited by extreme temperature-induced overwintering diapause. Lack of winter diapause allows perennialism, with nest founding and late stage male production in the population throughout the year (West-Eberhard, 1969). We discovered and validated set of single-nucleotide polymorphism (SNP) markers, with crossover between *P. canadensis* and *P. lanio*, to study the breeding and mating systems of these two species. Estimating the average relatedness of male offspring to other brood, and comparing it to that of female offspring to brood, can help reveal whether males may stand to gain comparable indirect fitness gains as females and thus if they may be predicted to mimic sister helping behaviour for the same ultimate payoffs. New foundress nests of *P. canadensis* were found to be established by highly related groups of either a renesting queen and her daughters, or groups comprised of full- and half-sisters. High relatedness was maintained in the nesting cycle. Post-emergence nests of *P. canadensis* and *P. lanio* that produced both female and male offspring had high reproductive skew, with a single queen usually the mother of nestmates. Natal males are likely to be fully related to siblings with shared parentage, thus there are opportunities for behaviours such as helping that increase indirect fitness.

1.9.2. Chapter 4: Is there potential assured male fitness returns through female longevity?

The original aim of this chapter was to setup laboratory breeding groups of tropical *P. canadensis*. However, long-term captivity in this genus is known to create behavioural artefacts (Jandt et al., 2015), and therefore this aim was abandoned. An outcome of captive

wasp data collection was the recording of longevity patterns in *P. canadensis* females. Nests of *P. canadensis* were collected from Panama and transferred into a captive laboratory environment in the United Kingdom. Females *P. canadensis* lived on average shorter than most bee and ant queens, but had the potential to live longer than most recorded wasp and some bee species. Variation in longevity was found between nests, and could possibly be attributed to unrecorded genetic or developmental factors linked to nutrition (Harvey et al., 2012; Gems & Partridge, 2013). Adult hymenopteran males are typically short-lived in comparison to females, expressing 'live-hard, die young' life history strategy (Heinze, 2016). The longevity of males may be extended through sperm stored in the spermatheca of long lived reproductive queens. Long-term sperm storage in females allows production of many daughters over her lifetime, that are directly related to the male. An alternative benefit of long lived females from the male's perspective is that fitness returns in male natal helping are assured; once a male departs the nest or dies, his investments as a helper are only assured if his sisters live long enough to rear and defend the brood (part-raised by males) to adulthood. Assured fitness returns (achieved by their natal nests being long-surviving) as an explanation for male helping behaviour fits the current for the evolution of altruism among female Hymenoptera as life insurers.

1.9.3. Chapter 5: What is the reproductive state of natal males, and how is dispersal regulated?

In the social Hymenoptera, males typically depart from natal nests sooner after pupal emergence to mate, whilst females may stay and help raise siblings. Male sex-biased dispersal, and its potential hormonal regulation, are investigated in *P. lanio*. Observations of adult male dispersal rates showed an association between age and natal departure. Through collection and dissection of males from natal nests, signs of developmental reproductive maturity were shown to increase with age. Further, in comparing the reproductive maturity of males present on the natal nest to those departed, natal males were less reproductively mature than those who had dispersed. Juvenile hormone is a potential master regulator of maturation and behaviour in male Hymenoptera. Newly emerged males given the juvenile hormone analog methoprene departed natal nests earlier, and had accelerated accessory gland swelling (a sign of reproductive maturity). Males therefore emerge from pupation reproductively immature, and spend time on natal nests before ultimately dispersing, with departure being regulated by juvenile hormone. These results set the stage for Chapter 6: male helping behaviour may represent a life history period whereby indirect fitness benefits are exploited whilst sexual maturation takes place; once mature, males disperse to invest in direct fitness.

1.9.4. Chapter 6: Do natal males help, and if so is there variance in helping effort associated with age and maturity?

There are reports of male helping-like behaviours across the Hymenoptera, however these behaviours have rarely been explored systematically through experimentation. Potential helping behaviour of natal *P. lanio* males was explored, using food distribution as an indicator of overt helping behaviour. Food distribution was found to occur when males received food from a returning foraging female, with males subsequently distributing food to brood. By experimentally offering males a standardised food source each day till departure, helping effort by food distribution was found to decrease with age. Contrary to expectations, no decrease in helping effort with increasing reproductive maturity was found. Helping behaviour may be regulated by more complex means, such as an interplay between maturation and juvenile hormone. Helping effort is likely to be more prominent in young males, as a 'making the best of the current situation' response to not being able to mate. When males age, there may be strong selection to stop helping and depart the nest to find mating opportunities, as the ultimate payoffs from direct fitness outweigh those achieved via indirect fitness by helping. Males therefore may be more involved in social dynamics and nest function (at least tropical *Polistes* paper wasps) than originally perceived.

Chapter 2: High indirect fitness benefits for helpers throughout the nesting cycle in a Neotropical eusocial paper wasp *Polistes canadensis*

Contributions: R. Southon, E. Bell, A. Radford, & S. Sumner designed the study; R. Southon & E. Bell conducted the fieldwork, with help from field assistant D. Fabbro; P. Graystock & C. Wyatt identified SNPs from transcriptome RNA-Seq assemblies; E. Bell conducted sample genotyping and dissections for foundress associations (part of this work went towards the thesis by E. Bell: Phenotypic plasticity and the evolution of castes in eusocial insects); R. Southon conducted overall SNP validation, sample genotyping and dissections for post-emergence nests, and analyses (whole dataset analysed, including previous foundress association and microsatellite genotypes); data of previous microsatellite genotypes for reanalysis provided by T. Lengronne & S. Sumner.

2.1. Abstract

Reproductive division of labour is a defining characteristic of eusociality in the Hymenoptera (ants, bees, and wasps), with individuals specialising in reproductive ('queen') or non-reproductive ('worker') roles ('castes'). The cosmopolitan *Polistes* paper wasps are popular models for studying how and why castes evolve, as they possibly share similar traits of the early ancestral stages of group living in the Hymenoptera, where females behave like workers but retain the ability to reproduce. To date, studies on social behaviour in *Polistes* are limited almost exclusively to temperate species. However, *Polistes* originated in the tropics, where (unlike for temperate species) seasonal constraints on reproductive options are weak and nests are effectively perennial. Here we present the first comprehensive study of genetic structure in a tropical *Polistes*. We discover and develop markers for 64 single-nucleotide polymorphism loci (SNPs) for *Polistes canadensis*, and used them to examine nesting group genetic structure and reproductive partitioning in both founding and post-emergence nests. Analyses reveal that adult co-foundresses are highly related, and reproduction is monopolised by a single female in established post-emergence nests (male and female offspring relatedness is at a relative maximum). This suggests that the potential indirect benefits from helping for female (and potentially male) offspring remain constant throughout the nesting cycle. These findings stress the potential influence of life history and ecology in the evolution of reproductive strategies.

2.2. Introduction

Societal living entails both benefits and costs for individuals (Krause & Ruxton, 2002). Unless clonal, one such cost is the reproductive conflict that exists between group members if only some individuals produce young (West et al., 2002). In extreme cases, only one or a few group members reproduce while other (non-reproducing) individuals assist in the rearing of offspring, or are forced to disperse to establish new groups to reproduce. Such division of labour and high reproductive skew is found in a range of social taxa (Wilson, 1971; Kokko & Johnstone, 1999; Cant & Johnstone, 2008). Determining how reproduction is divided amongst conspecific group members, and why individuals forgo independent breeding or disperse to found new groups, is fundamental for understanding the evolution of sociality (Keller & Reeve, 1991; West et al., 2007).

Helping may evolve because of direct and/or indirect fitness benefits. Where group relatedness is low or less than the population average, as in many cooperatively breeding mammals and birds, helping can be explained by immediate or delayed direct benefits. These include the advantages accrued from group augmentation or the potential to inherit the breeding position or territory (Clutton-Brock, 2009). When group members are closely related, as in the complex eusocial Isoptera and Hymenoptera, the cost of helping can be countered by indirect (kin-selected) benefits (Queller & Strassmann, 1998), as first proposed by Hamilton (1964). Changes in group composition that alter average group relatedness have potential implications for the relative payoffs of helping versus reproducing. Such changes can arise due to both intrinsic factors (e.g. intra-group competition and turnover of breeders) and extrinsic factors (e.g. seasonal constraints and fluctuating resource availability) (McCracken & Wilkinson, 2000; Koštál, 2006; Armitage, 2007; Wittemyer et al., 2007). Tests of how environmental variation influences the genetic structure of groups, and the resulting fitness payoffs, are needed for a full understanding of the relative importance of direct and indirect fitness for helpers (Bourke, 2014).

The simple eusocial societies of *Polistes* paper wasps (where there is division of labour in behavioural roles, but all individuals retain the potential to reproduce) share similar traits to that of the early ancestral stages of eusocial evolution (West-Eberhard, 1969; Jeanne, 1980; Reeve, 1991; Danforth & Eickwort, 1997; Keller, 2003). Studies to date have indicated the importance of both direct and indirect fitness benefits for *Polistes* sociality (Queller et al., 2000; Boomsma, 2007; Leadbeater et al., 2011; Field & Leadbeater, 2016). For example, in the best-studied species of the genus, *Polistes dominula*, direct fitness via nest inheritance has been shown to explain helping behaviour of females during the group-founding stage, when group members are often unrelated (Strassmann et al., 1989; Kokko & Johnstone, 1999; Queller et al., 2000; Zanette & Field, 2008; Leadbeater et al., 2011; Field & Leadbeater, 2016). By contrast, indirect fitness from raising siblings explains helping behaviour of females in later

nesting stages of *P. dominula*, where the mothers of the helpers monopolise reproduction resulting in high sibling relatedness (Peters et al., 1995; Queller et al., 1997; Field et al., 1998; Queller & Strassmann, 1998; Reeve & Keller, 2001; Seppä et al., 2002). For *Polistes* (as for other hymenopterans), the high direct fitness payoffs as a haploid male from investing in mating and the production of highly related daughters (r = 1.0) likely explains lack of helping behaviour in males (Hamilton, 1972; Boomsma, 2007).

Inclusive fitness theory states individuals should stay and help if gains through natal philopatry (indirect fitness via helping; direct fitness via sneak subordinate egglaying or inheritance) outweigh dispersing to produce own offspring (Hamilton, 1964, Queller & Strassmann, 1998; Leadbeater et al., 2011). However, as hymenopteran males are haploids and females are diploids, asymmetries may exist in relation to predicting which sex stays or disperses from the nest – based on relatedness values to nestmates. For example, in newly emerged female offspring reduced relatedness to brood from additional egglayers or multiple mating of the mother may decrease both indirect fitness gains from helping and direct fitness gains through nest inheritance, and result in direct fitness gains through egglaying on the natal nest or dispersing becoming the primary fitness strategy; in newly emerged males, reduced relatedness to brood will not influence ultimate dispersal to mate, as it will always be the optimal payoff for males (Hamilton, 1972). However, if male helping is to occur (for example, if dispersal conditions are temporarily suboptimal, see Chapter 5), we may expect it to be beneficial to males (if the behaviour is related to indirect fitness gains) when relatedness values to brood align with that female helpers because costs verses payouts may be similar.

Species such as *P. dominula* are temperate and experience obligate winter diapause (or quiescence), which has a profound impact on group dynamics: payoffs to female helpers from indirect fitness in founding nests may be uncertain, because it is difficult for females emerging from diapause to find and select relatives with which to found a nest (Queller et al., 2000; Starks, 2003; Dapporto et al., 2004; Zanette & Field, 2008; Field & Leadbeater, 2016); payoffs for direct fitness in short-lived males are seasonally assured by a mating window (i.e. receptive females in the population) (Lucas & Field, 2013). In the tropics, insects are not subject to the same extreme temperature-induced overwintering diapause periods as their temperate counterparts (Denlinger, 1986). This environmental variation means that nests of tropical Hymenoptera can be essentially perennial, and individuals can found nests, produce sexual females and males, and thus mate at any time of the year (West-Eberhard, 1969; Pickering, 1980; O'Donnell & Joyce, 2001). The availability of mates all year round (for both males and females) opens options for reproductive strategies; for example, females are not limited by the availability of males in their decision to found their own nests as a mated queen; likewise, males are not limited by a specific window of time when mating is profitable (e.g. mating flights or leks). *Polistes* originated in the tropics (Carpenter, 1996; Santos et al., 2015) and two-thirds of species in this genus are tropical (West-Eberhard, 1969). Yet we lack data

on the fitness strategies employed in tropical *Polistes* species (Strassmann et al., 1989), which are likely to experience ecological conditions similar to those under which sociality first evolved.

Here we examine nesting group genetic structure and reproductive partitioning in the simple eusocial societies of the tropical paper wasp, *Polistes canadensis*, which is emerging as a key model for tropical *Polistes* research (West-Eberhard, 1969; Jeanne, 1979; Pickering, 1980; Sumner et al., 2006; Sumner et al., 2007; Ferreira et al., 2013; Patalano et al., 2015). New nests are co-founded by several females (West-Eberhard, 1969; Pickering, 1980), yet it is unknown whether these females are often unrelated, as in *P. dominula* (in one study, 15–35% of non-reproductive co-foundresses were unrelated to the dominant reproductive in groups – Leadbeater et al., 2011). Additionally, it is unknown whether subordinate workers lay some of the male eggs, the latter of which would reduce natal male relatedness to the nest (i.e. between queen and worker male offspring), and potentially impact the presence of male helping (if helping relies on relatedness outweighing cost) (Arévalo et al., 1998; Strassmann et al., 2003; Tsuchida et al., 2004; Chapter 1, Table 1.1). The República de Panamá, in the central range for *P. canadensis*, experiences seasonal variation in the form of wet and dry periods, but there is no enforced overwintering diapause period. All stages of the nesting cycle can be found together throughout the year (including the dry season) (West-Eberhard, 1969; Pickering, 1980), permitting their simultaneous study under the same environmental conditions. Taken together, these life history and environmental traits allow us to investigate whether populations that lack diapause consist of highly related co-foundresses, low conflict over reproduction, and thus high indirect fitness payoffs to helpers throughout the nesting cycle. Support for this hypothesis would imply that direct fitness may be less important in the early stages of social evolution when environmental conditions have little effect on group membership and genetic structure.

Microsatellite markers are still widely popular in mapping genetic relatedness structures of populations, due in part to a low number of highly informative and polymorphic markers allowing for relatively low genotyping costs (Abdul-Muneer, 2014). However, highly polymorphic microsatellite markers are prone to high mutation rates that are difficult to predict, reducing marker effectiveness at detecting heritable alleles (Morin et al., 2004). Additionally, comparisons of microsatellite studies from different research groups may suffer from variation in allele calling techniques and errors in size determination. Single-nucleotide polymorphism (SNP) markers can be used as an alternative, given that enough low-informative bi-allelic markers are utilised to match the information provided by highly polymorphic microsatellites (Hauser et al., 2011; Gärke et al., 2012; Fernández et al., 2013). SNPs have the advantage of low genotyping assignment errors and ease in cross-study/laboratory comparisons (Hauser et al., 2011). Furthermore, in the study of Hymenoptera populations with diploid females and haploid males, SNPs may provide an

advantage in sex determination of ambiguous brood (e.g. eggs) and in male parental analysis. Sex of ambiguous brood in microsatellite studies of Hymenoptera is usually determined by a male being called as possessing a fully homozygous genotype (in fact a hemizygous genotype). However, due to the low number of markers utilised, there can be errors in determining if an individual is a true hemizygote or female homozygote at all the observed loci (Chapman & Crespi, 1998). Errors in SNP-assisted sex determination should theoretically be lower, as more alleles are genotyped thereby reducing the chance of a female being called a homozygote at every locus. Likewise, high allele coverage in SNPs may facilitate hymenopteran male pedigree construction. If the nesting queen and her adult offspring (e.g. workers) both lay male eggs, as male offspring only inherit one of their mothers' alleles at a locus (there are no direct paternal alleles), then there is a high chance that all the alleles called will be shared by both the queen and her adult offspring – reducing the accuracy of assigning the genotyped male to the true mother (Châline et al., 2002).

We first identified and optimised a set of SNP markers from existing transcriptome data (Patalano et al., 2015); these markers permit the fine-scale resolution of genetic structure required to estimate fitness payoffs. Then, we used these markers to test three hypotheses. Hypothesis 1: Adult co-foundresses are close relatives, which would suggest high indirect fitness payoffs for female joiners. Hypothesis 2: There is an effective monopoly of reproduction (i.e. high reproductive skew) in foundress association nests, meaning that there are high indirect fitness payoffs for non-reproductive (related) co-founders. Hypothesis 3: A reproductive monopoly is perpetuated in post-emergence nests, assuring long-term: direct and indirect fitness payoffs for females (singly mated, single egglaying mother on the nest) via nest inheritance and helping; potential indirect fitness payoffs for male helpers (single egglaying mother on the nest). Finally, we reanalysed previous microsatellite genotypes (that had only reported general population structure using five female pupae per nest) from nests of *P. canadensis* in a nearby population to confirm our SNP results (Lengronne et al., 2012).

2.3. Methods

2.3.1. SNP-marker discovery and validation

SNPs were identified from transcriptome RNA-seq assemblies available for ten individuals of *P. canadensis* (phenotypes of four queens and six workers) (Patalano et al., 2015), and for unpublished transcriptome sequences for ten individuals of a sister species *Polistes lanio* (phenotypes of five queens and five workers) collected at Verdant Vale, Blanchisseuse Rd, Trinidad, Trinidad & Tobago (10°41'5.44"N, 61°17'24.95"W) (Moreno et al., in prep.). Raw RNA-seq reads were mapped against the *P. canadensis* genome (Patalano et al., 2015) using the Burrows–Wheeler Aligner to obtain binary alignments maps (BAMs), sorted using

SAMtools, and then SNPs were called using mpileup and view functions within the BCFtools package (Li et al., 2009; Li, 2011).

To identify SNP sites that were likely to be common across *P. canadensis* and not just from the sampled population, and that generated a more broad-reaching resource for *Polistes* SNP analysis, biallelic SNP sites were selected from those found in both species. Quality filtering of candidate SNPs was performed by visualising against RNA-seq assemblies of *P. canadensis* and *P. lanio* using Integrative Genomics Viewer (IGV) (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). SNPs with low allele frequencies (< 20%) or a sequencing depth fewer than 150 reads, were filtered out. As an additional quality filter, and to aid the reliability of the downstream KASP™ (LGC Genomics) genotyping assays, only SNPs located more than 80 bases from one another or to the end of a scaffold were selected. From this process, 120 SNPs were selected across a range of scaffolds with the highest coverage.

A conservative filtering method was used to select a set of 64 polymorphic SNP loci for KASP™ SNP genotyping from the 120 candidate SNPs (see 2.3.4. Genotyping). To identify a set of loci that were reliably polymorphic for this species and study population, initial validation using 120 identified loci was performed on a subset of 55 individuals from across five post-emergence nests. Loci were selected through manual examination of clusters, removing loci with monomorphic or indeterminate allelic clustering, along with removal of any loci with Minor Allele Frequencies (MAF) < 5%. Subsequently, 69 loci were selected for the full genotyping project. The guidelines of Semagn et al. (2014) were followed in removing individual ambiguous allele calls to reduce genotyping error. To test for deviations from Hardy–Weinberg equilibrium (Lange et al., 2005) and linkage disequilibrium by Fisher's exact test between loci pairs (Lazzeroni & Lange, 1998), 40 females from separate nests (see 2.3.2 Genetic-structure sample collection) were tested using the software Mendel 14.5 (Lange et al., 2013). Loci with deviations from Hardy–Weinberg equilibrium were removed. The final set of 64 SNP loci were used in all subsequent genetic-structure analyses and testing of our hypotheses.

2.3.2. Genetic-structure sample collection

We analysed both foundress and post-emergence nests of *P. canadensis* with a range of comb sizes and brood compositions (nesting group characteristics are given in Table 2.1). Foundress associations were defined as nests containing only eggs or small larvae, with no evidence of emerged adults (i.e. hatched pupae caps), and with at least four adult females present. Post-emergence nests were defined as those with a mixture of developing brood (eggs, larvae, and pupae), and evidence of hatched pupae caps.

All nesting groups were collected in May–August 2013 from abandoned buildings at a four hectare site at Fuerte Sherman, Colón Province, República de Panamá (9°21'42.57"N, 79°56'58.49"W). Twenty nesting groups (ten of each stage) were selected for SNP genotyping. All nests consisted of single combs (multiple combs did not exist in this population, pers. obs.); nests with satellite combs (a new comb started by nesting group members within immediate vicinity – Jeanne, 1979) were excluded from selection by avoiding selection of nests with combs within 50 cm of the main nest. Nests in which brood parasitism was detected (e.g. Ichneumonoidea pupae capped cells) were also excluded. Prior to the collection of post-emergence nests, two female phenotypes were identified from behavioural observations to minimise the number of adult females required for genotyping. The queen was identified on each nest by removal of an egg in a cell and observing which individual subsequently oviposited within an hour (Sumner et al., 2006). A worker was identified through *ad libitum* observation of the nest, noting if a wasp brought a solid mass of forage to the nest. To enhance statistical power in SNP-marker validation (see 2.3.1. SNP-marker discovery and validation), a single female was collected from each of 20 additional post-emergence nests on the site. Nests (with adults and brood) were collected at dusk, when most foraging wasps are likely to have returned to the comb. Samples were stored in 80% EtOH at -20°C. The number of cells and brood (categorised as eggs, larvae, or pupae) were counted in all sampled nests.

Table 2.1. Nesting group characteristics for foundress and post-emergence nests (sampling for SNP analysis).

ID & Assignment		Genotyping Method	Nesting Group Characteristics								
			Number of Adults on Nest	Number of Dev. Offspring in Nest	Size of Comb (Total Number of Cells)	Number of Eggs in Nest	Number of Larvae in Nest	Number of Pupae in Nest	Number of Empty Cells in Nest	Number of Parasitised Cells in Nest	Ratio of Adult-Larva in Nest
FA #01	Mixed	SNPs	5	12	12	12	0	0	0	0	-
FA #02	Matrilineal	SNPs	4	10	20	10	0	0	10	0	-
FA #03	Matrilineal	SNPs	9	22	29	22	0	0	7	0	-
FA #04	Sister	SNPs	4	19	19	19	0	0	0	0	-
FA #05	Sister	SNPs	6	13	14	12	1	0	1	0	-
FA #06	Sister	SNPs	4	24	24	24	0	0	0	0	-
FA #07	Sister	SNPs	8	15	37	15	0	0	22	0	-
FA #08	Sister	SNPs	12	15	20	15	0	0	5	0	-
FA #09	Sister	SNPs	9	27	35	25	2	0	8	0	-
FA #10	Unknown	SNPs	4	11	17	11	0	0	6	0	-
Mean ± SE FC (SNPs)		SNPs	6.50 ± 0.90	16.80 ± 1.86	22.70 ± 2.68	16.50 ± 1.77	0.30 ± 0.21	0.00 ± 0.00	5.90 ± 2.14	0.00 ± 0.00	-
Post-emergence #01		SNPs	43	268	285	70	132	66	17	0	0.33
Post-emergence #02		SNPs	23	92	178	30	7	55	86	0	3.29
Post-emergence #03		SNPs	23	269	300	70	140	59	31	0	0.16
Post-emergence #04		SNPs	41	417	425	129	241	47	8	0	0.17
Post-emergence #05		SNPs	80	409	1218	122	132	155	809	0	0.61
Post-emergence #06		SNPs	23	172	186	17	137	18	14	0	0.17
Post-emergence #07		SNPs	32	197	203	74	72	51	6	0	0.44
Post-emergence #08		SNPs	30	138	213	44	45	49	75	0	0.67
Post-emergence #09		SNPs	27	156	198	49	55	52	42	0	0.49
Post-emergence #10		SNPs	14	144	145	54	58	32	1	0	0.24
Mean ± SE Post- (SNPs)		SNPs	33.60 ± 5.85	226.20 ± 35.69	335.10 ± 101.41	65.90 ± 11.46	101.90 ± 21.37	58.40 ± 11.57	108.90 ± 78.33	0.00 ± 0.00	0.66 ± 0.30

2.3.3. Assessment of female reproductive state

All adult females were dissected to estimate reproductive status (Cini et al., 2013). Ovarian development of each individual was assigned to one of five categories (adapted from Gobbi et al., 2006): A = small filamentous ovarioles lacking oocytes; B = small ovarioles with slightly developed oocytes; C = large ovarioles with few developed oocytes at the base of tract; D = large ovaries with multiple fully developed oocytes; and E = large ovaries with visible regression. The length of the largest oocyte in the reproductive tract was recorded in millimetres, and considered a mature egg if over 2 mm in length (Sumner et al., 2006). We defined reproductively capable females as having mature eggs (category C–E), and actual egglaying females as having mature eggs and a maternal genotype that is compatible with being the mother of the brood's genotypes (see 2.3.5. Nesting group genetic structure analyses) (Cini et al., 2013). To test whether more subordinate co-foundresses (i.e. those without genotyped offspring on the nest) were reproductively capable than not, we used a paired sample t-test, pairing number of individuals per nest with mature eggs (category C–E) and those without (A–B).

2.3.4. Genotyping

To determine the genetic structure of foundress and post-emergence nests, we utilized our new SNP markers (64 SNP loci). For the ten foundress associations, all adults and 25–80% of eggs were genotyped (65 adults and 72 eggs total; Appendix I). For the ten post-emergence nests, we selected the reproductive and foraging females that were identified from observations (2.3.2. Genetic-structure sample collection), together with five eggs and up to five pupae of each sex per nesting group (10 reproductives, 10 foragers, 50 eggs, 50 female pupae and 39 male pupae total; Appendix I). For the post-emergence nests, eggs and pupae were sampled from across the nest by dividing a comb into quarter grids, and selecting across grids in sequence until the required sample size was collected (or no more eggs/pupae were present). Sampling across the comb in this way reduced the chances of biasing the results if, for example, different individuals dominated reproduction in specific areas of the nest (as suggested in West-Eberhard, 1986). Adults were sexed based on reproductive physiology. Pupae were sexed by counting the number of antennal flagellum segments; those with 10 segments were scored as females and those with 11 as males (Strassmann et al., 2003).

Prior to DNA extraction, samples were washed in 90% EtOH to reduce the chance of foreign DNA contamination on the exoskeleton (Shokralla et al., 2010), and allowed to dry at room temperature. For adults and pupae, 2 mm of coxa was dissected and broken apart to reveal tissue. Eggs were removed intact from cells. DNA was extracted using a HotSHOT protocol (Truett et al., 2000; Montero-Pau et al., 2008), with individual coxae and eggs added to 50 µl alkaline lysis buffer (NaOH 50 mM, disodium EDTA 0.4 mM, pH 12.0) and heated to 95°C for 2 h. In neutralisation, 50 µl of Tris-CHl 40 mM pH 5.0 was added, and a solution created of 35 µl

DNA extract diluted with 100 µl H_2O. Extracted DNA was stored at 4°C (short-term) or -20°C (long-term) before use in PCR reactions.

SNP genotyping was conducted using KASP™ genotyping assays. Reaction master mixes consisted of 11.83 µl custom KASP™ assay mix, 422.4 µl KASP™ V4.0 2x Master Mix v4.0 low ROX™, and 422.4 µl H_2O added in 8 µl aliquots to 2 µl of each extracted DNA sample, and spun for 4 min at 2500 rpm. Thermal cycling was performed with an Agilent Mx3005P qPCR System and consisted of 94°C for 15 min (1 cycle); 94°C for 20 s and 61°C for 1 min (10 cycles); 94°C for 20 s and 55°C for 1 min (35 cycles); finally, 30°C for 1 min. Pre- and post-cycle fluorescence was read at 25°C, with dyes HEX™, FAM™, and ROX™ reference dye, normalised and plotted in MxPro™ Mx3005P® v4.10. Between 0 and 5 additional cycles of 94°C for 20 s and 55°C for 1 min were performed after manually evaluating 35 cycle touchdown post-PCR reads. Each PCR plate included at least three negative (no DNA) and two positive (duplicates for initial runs, repeats for subsequent runs) controls. Pre-extraction sexing of adults and pupae allowed checking of genotyping accuracy through sex-specific hemi- (male) and hetero- (female) zygosity of the haplodiploid genetic system that Hymenoptera exhibit. No diploid males were detected amongst pupae (Liebert et al., 2004); we used the criteria set of < 2% heterozygosity across loci in a sample to identify male eggs.

2.3.5. Nesting group genetic structure analyses

Female-only pairwise relatedness was estimated using COANCESTRY 1.0.1.5 (Wang, 2011), reporting the Wang (2002) relatedness estimation. Mean relatedness across groups was calculated using the mean of each group, so each group had equal weighing irrespective of the number of individuals sampled (Lengronne et al., 2012). Pairwise relatedness of individuals within groups was plotted against simulated populations consisting of unrelated ($r = 0$), cousin ($r = 0.1875$), and full-sister ($r = 0.75$) pairs (pairwise relatedness to 1 d.p.). Simulated populations were created with KINGROUP v2_090227 (Konovalov et al., 2004), based on the observed allele frequencies of females in the SNP genotyped population (using the Queller & Goodnight relatedness estimation (Queller & Goodnight, 1989)). Individual F_{IS} inbreeding coefficients of females in nests were derived from the Lynch & Ritland (1999) estimate calculated in COANCESTRY, and tested for significant differences from a coefficient of zero (no significant inbreeding) using one-sample t-tests for the two stages of the nesting cycle (foundress and post-emergence).

Female and male pairwise relatedness was calculated for offspring on post-emergence nests (SNP dataset), using PolyRelatedness v1.6, reporting the Huang et al. (Huang et al., 2014) method-of-moments relatedness estimation. The regression relatedness and a simulated life-for-life relatedness (dividing the regression relatedness of sisters to brothers by two, and multiplying the regression relatedness of brothers to sisters by two) was plotted for each sex

to other female and male siblings, along with the hypothetical relatedness to the nest if equal sex ratios are presumed (calculating the overall mean relatedness from each sex).

Adults and brood were assigned to matrilines, and the genotypes of their putative mothers (matrilines) and fathers (patrilines) were predicted based on allele sharing, using COLONY 2.0.5.8 (Jones & Wang, 2010). The predicted genotypes were then compared with the observed genotypes of sampled adults; if a predicted genotype matched the observed genotype of an adult female with mature ovaries, we assumed that this was the mother on the nest. If more than one female per nest fitted this criterion, we did not assign the brood to a specific female. A full-likelihood method using all genotypes in a single model was used, and assignment of clusters accepted only when the probability of assignment was ≥ 0.80. We allowed for the possibility of polyandry in matriline assignment, as our preliminary analyses suggested a low level of multiple mating was present (see 2.3.6. Mating frequency). The protocol for matriline assignment consisted of creating a prior model with all adults included as potential mothers to brood; any adults not assigned as potential mothers to brood were reanalysed as potential offspring to recognised mothers.

Reproductive skew indexes give a measure of reproductive partitioning among potential parents. We used the B index to measure reproductive skew of matrilines (i.e. in genotyped offspring), a binomial index combining observed variance and expected variance, scaling with group size (Nonacs, 2000). Although there is no consensus on a single best skew index, the B index has flexible assumptions and allows for small sample sizes (Nonacs, 2003). Calculated reproductive skew in a group may range from -1 (equally shared) to 1 (monopolised) (see formula in Nonacs, 2000). The B index was determined in SKEW CALCULATOR 2003 © Peter Nonacs with 1000 simulations, 95% confidence intervals, $\alpha = 0.05$. The default setting of equal length of time spent in the nesting group per potential mother was assumed, as the actual amount of time adult females were in the group for was unknown.

2.3.6. Mating frequency

We report the effective mating frequency of females k_{e3} (Nielsen et al., 2003) across matrilines (i.e. number of fathers expressed in genotypes), excluding any matrilines consisting of a single genotyped individual. Effective mating frequencies were then compared to a k_{e3} of 1 (singly mated) and 2 (twice mated) using a one-sample t-test. We then combined effective mating frequency (k_{e3}) data from all applicable matrilines across successfully assigned nests (in COLONY, including microsatellite data below, see 2.3.7. Confirming SNP results with previous microsatellite data), and tested whether effective mating frequency in this population differed significantly from 1 (singly mated) and 2 (twice mated) using a one-sample t-test.

2.3.7. Confirming SNP results with previous microsatellite data

We reanalysed published microsatellite data for 24 post-emergence colonies of *P. canadensis* (Lengronne et al., 2012): matriline and patrilines were constructed in COLONY, with matriline reproductive skew (B index), and effective mating frequency (k_{e3}) reported, as per the SNP dataset (see Table 2.2 for nesting group characteristics). Five female pupae sampled per nest; nests were located 9.6 km away from our SNP sample site across the Canal de Panamá at Punta Galeta, Colón, República de Panamá (9°24′08.28′′N, 79°52′19.41′′W). We compare post-emergence nest B skew index between SNP and microsatellite samples using a one-sample t-test.

General Statistical Protocol: All statistics, unless otherwise stated, were performed in R 3.2.1 (R Core Team, 2013) to $\alpha = 0.05$, reporting the mean ± SE.

Table 2.2. Nesting group characteristics for post-emergence nests (microsatellite sampling – Lengronne et al., 2012)

ID & Assignment	Genotyping Method	Nesting Group Characteristics								
		Number of Adults on Nest	Number of Dev. Offspring in Nest	Size of Comb (Total Number of Cells)	Number of Eggs in Nest	Number of Larvae in Nest	Number of Pupae in Nest	Number of Empty Cells in Nest	Number of Parasitised Cells in Nest	Ratio of Adult-Larva in Nest
Post-emergence #11	*Microsats.*	24	232	233	68	102	62	0	1	0.24
Post-emergence #12	*Microsats.*	41	310	317	106	134	70	7	0	0.31
Post-emergence #13	*Microsats.*	13	117	117	48	50	19	0	0	0.26
Post-emergence #14	*Microsats.*	13	176	177	57	95	24	0	1	0.14
Post-emergence #15	*Microsats.*	22	276	277	82	161	33	0	1	0.14
Post-emergence #16	*Microsats.*	17	180	187	78	88	14	7	0	0.19
Post-emergence #17	*Microsats.*	19	215	216	86	97	32	1	0	0.20
Post-emergence #18	*Microsats.*	20	389	391	135	174	80	2	0	0.11
Post-emergence #19	*Microsats.*	22	190	190	40	119	31	0	0	0.18
Post-emergence #20	*Microsats.*	34	351	351	114	161	76	0	0	0.21
Post-emergence #21	*Microsats.*	15	162	162	42	90	30	0	0	0.17
Post-emergence #22	*Microsats.*	22	161	200	42	69	50	39	0	0.32
Post-emergence #23	*Microsats.*	4	51	51	25	21	5	0	0	0.19
Post-emergence #24	*Microsats.*	6	76	79	33	34	9	3	0	0.18
Post-emergence #25	*Microsats.*	13	110	110	81	12	17	0	0	1.08
Post-emergence #26	*Microsats.*	38	295	296	104	149	42	1	0	0.26
Post-emergence #27	*Microsats.*	7	60	60	10	40	10	0	0	0.18
Post-emergence #28	*Microsats.*	10	128	129	17	83	28	0	1	0.12
Post-emergence #29	*Microsats.*	11	94	101	29	56	9	7	0	0.20
Post-emergence #30	*Microsats.*	9	70	70	35	26	9	0	0	0.35
Post-emergence #31	*Microsats.*	30	302	316	89	153	60	13	1	0.20
Post-emergence #32	*Microsats.*	16	109	144	30	53	26	35	0	0.30
Post-emergence #33	*Microsats.*	33	149	253	26	43	80	104	0	0.77
Post-emergence #34	*Microsats.*	12	93	94	48	29	16	0	1	0.41
Mean ± SE Post- (*Microsats.*)	*Microsats.*	18.79 ± 2.09	179.00 ± 19.89	188.38 ± 20.07	59.38 ± 6.96	84.96 ± 10.26	34.67 ± 5.00	9.13 ± 4.64	0.25 ± 0.09	0.28 ± 0.04

2.4. Results

2.4.1. SNP-loci discovery and validation

Of the 20,402 SNPs identified, 1,790 were heterozygous. Amongst these SNPs, 918 had allele frequencies > 20%, with an even coverage against both *P. canadensis* and *P. lanio* assemblies. After proximity filtering, 202 high-quality SNP candidates remained. The final 120 SNPs were selected from these for validation, based on coverage (minimum 765 reads) and location across a range of 80 scaffolds.

Initial selection with KASP™ genotyping (120 loci) identified 25 loci for removal that failed manual clustering evaluation; a further two loci were excluded due to MAFs less than 5%; another 24 loci were excluded due to poor genotyping clarity. This filtering left 69 loci. Significant deviations from Hardy–Weinberg were detected in five loci and these loci were removed. A final 64 loci had MAFs > 10% (62 loci) and >5% (two loci), with linkage disequilibrium (Fisher's exact test) observed across 88 pairs of loci (4.2% of pairings). Genotyped samples had no discrepancy with positive controls. The percentage of successful manual allele assignment within samples was 78.9% ± 0.7% (in 64 validated loci). These 64 loci were used for full genetic-structure analyses in the ten foundress and ten post-emergence nests (Appendix II).

2.4.2. Nesting group demographic and genetic characteristics

Foundress associations consisted of 6.5 ± 0.9 adults (all female), with combs of 22.7 ± 2.7 cells. Post-emergence nests consisted of 33.6 ± 5.9 adults (mixed sex, at least a small proportion of adult males per nest), with combs of 335.1 ± 101.4 cells. No male brood were detected in foundress associations; whilst there were male brood in post-emergence nests (in eight out of ten nests, male pupae where discovered, with male eggs discovered through genotyping in all ten nests – mean across nests, 56.0 ± 8.8% of genotyped eggs).

A female heterozygosity of H_{obs} = 0.426 ± 0.005 (range: 0.238–0.780) was observed across all loci. In foundress associations, F_{IS} = -0.084 ± 0.016 (lower/upper 95% CI: -0.115/-0.052), which was significantly different from 0 (one-sample t-test: t_{140} = -5.181, $p < 0.001$), suggesting some outbreeding. In post-emergence nests, F_{IS} = 0.010 ± 0.013 (lower/upper 95% CI: -0.015/0.035), which was not significantly different from 0 (t_{90} = 0.770, p = 0.443), suggesting no significant inbreeding or outbreeding.

2.4.3. Hypothesis 1: Foundress associations consist of close relatives

Co-foundresses were mostly close relatives. Mean relatedness (*r*) between adult female co-foundresses across all 10 foundress associations was 0.68 ± 0.05 (range: 0.48–0.84; Table 2.3). The highest frequency of calculated pairwise relatedness between adult females within foundress associations was at *r* = 0.8, with a secondary peak at *r* = 0.3 (Figure 2.1).

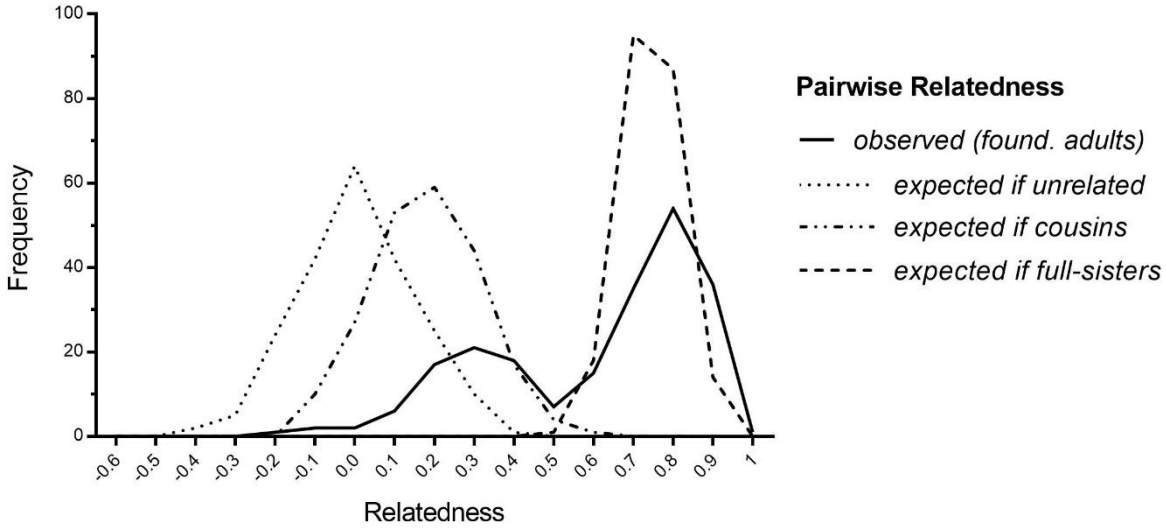


Figure 2.1. (*observed*) Pairwise relatedness distribution of adult co-foundresses within groups; (*expected*…) simulated population (215 pairs) if the foundress population consisted of individuals being *unrelated r* = 0, *cousins r* = 0.1875, or *full-sisters r* = 0.75.

COLONY analyses, which grouped individuals into sib-groups, revealed three types of family structure in foundress associations (Figure 2.2). Sib-grouping assignment among adults was successful (clustering probability > 0.80) in nine of ten foundress associations. Probability of assignment in both adults and brood was 0.91 ± 0.01 (foundress association (FA) #10 removed from adult analysis; referred to as '*Unknown*' in subsequent tests of brood). '*Sister*' associations best described the genetic structure in six associations. Three associations (FA #4–#6) consisted of a single matriline of sisters (foundress *r* = 0.82 ± 0.02). Three other association (FA #7–#9) consisted of two matrilines (foundress *r* = 0.53 ± 0.020); relatedness between matrilines in these association was *r* = 0.15 ± 0.08, 0.25 ± 0.02, and 0.36 ± 0.03 in FA #7–#9 respectively. The second type of genetic structure was '*Matrilineal*', in which a mother had renested (i.e. left the mature, post-emergence nest on which she was likely to have been queen) with daughters. '*Matrilineal*' associations were detected in two association (FA #2–#3;

r = 0.76 ± 0.01); daughters were related to their respective renesting mothers by r = 0.47 ± 0.06. The remaining foundress associations had three matrilines (defined as '*Mixed*'), with a mother, her three daughters, and an individual from a third matriline (FA #1; r = 0.49); this latter individual was related to the 'mother' by r = 0.39. The three renesting mothers across associations FA #1–#3 were likely unrelated (r = 0.04 ± 0.05).

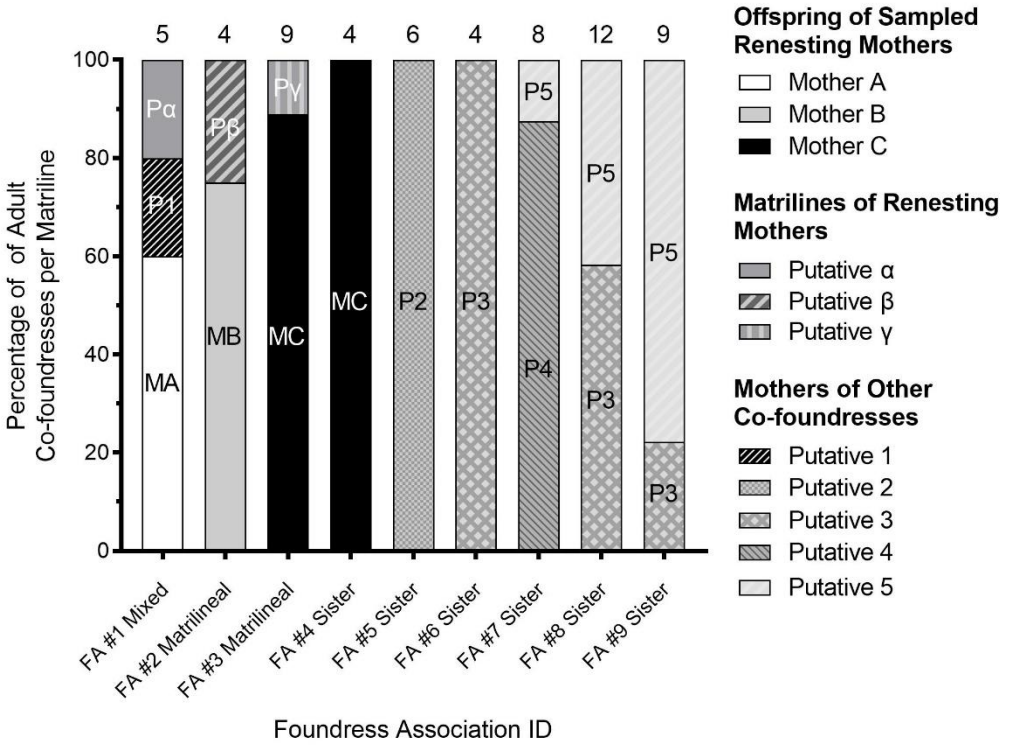


Figure 2.2. Percentage of adult females in foundress associations assigned to matrilines. Three types of genetic structure were identified: *Sister* (7 associations), *Matrilineal* (2 associations), and *Mixed* (1 association). Number of adults total in an association listed above each column. Legend indicates the mother 's identity for each matriline.

Table 2.3. Nesting group relatedness calculated using the Wang estimate (Wang, 2002) in COANCESTRY 1.0.1.5 (Wang, 2011); Maternity assignment constructed in COLONY 2.0.5.8 (Jones & Wang, 2010); and Reproductive skew represented as the B index (Nonacs, 2000), calculated in SKEW CALCULATOR 2003 © Peter Nonacs (SNP samples).

ID / Type		Genotyping Method	Mean Relatedness in Female Adults	Mean Relatedness in Female Brood	Number of Matrilines in Female Adults	Number of Matrilines in Female Brood	Number of Matrilines in Male Brood	Reproductive Skew in Brood (B index)
FA#01	Mixed	SNPs	0.49	0.79	3	1	*no male brood*	0.533
FA#02	Matrilineal	SNPs	0.77	0.59	2	2	*no male brood*	0.438
FA#03	Matrilineal	SNPs	0.75	0.83	2	1	*no male brood*	0.778
FA#04	Sister	SNPs	0.84	0.64	1	1	*no male brood*	0.656
FA#05	Sister	SNPs	0.83	0.70	1	1	*no male brood*	0.714
FA#06	Sister	SNPs	0.79	0.70	1	1	*no male brood*	0.656
FA#07	Sister	SNPs	0.53	0.76	2	2	*no male brood*	0.505
FA#08	Sister	SNPs	0.49	0.76	2	1	*no male brood*	0.802
FA#09	Sister	SNPs	0.57	0.70	2	1	*no male brood*	0.762
FA#10	Unknown	SNPs	0.76	0.76	*n/a*	1	*no male brood*	0.656
Mean ± SE FA (SNPs)		SNPs	0.68 ± 0.05	0.72 ± 0.02	1.78 ± 0.22	1.20 ± 0.13	*no male brood*	0.650 ± 0.039
Post-emergence #01		SNPs	0.50	0.72	2	1	1	0.912
Post-emergence #02		SNPs	0.50	0.74	2	1	1	0.893
Post-emergence #03		SNPs	0.38	0.71	2	1	1	0.893
Post-emergence #04		SNPs	0.53	0.74	2	1	1	0.911
Post-emergence #05		SNPs	0.64	0.73	2	1	1	0.922
Post-emergence #06		SNPs	0.56	0.76	2	1	1	0.861
Post-emergence #07		SNPs	0.63	0.63	2	2	1	0.692
Post-emergence #08		SNPs	0.46	0.73	2	1	1	0.902
Post-emergence #09		SNPs	0.51	0.82	2	1	1	0.899
Post-emergence #10		SNPs	0.51	0.73	2	1	1	0.867
Mean ± SE Post- (SNPs)		SNPs	0.52 ± 0.02	0.73 ± 0.01	2.00 ± 0.00	1.10 ± 0.10	1.00 ± 0.00	0.875 ± 0.021

2.4.4. Hypothesis 2: Reproductive monopoly in foundress associations

A single egg-layer was detected in most foundress associations. All genotyped brood were female. Mean relatedness among female brood within foundress associations was r = 0.72 ± 0.02 (range: 0.59–0.83; Table 2.3). The highest frequency of calculated pairwise relatedness between female brood within foundress associations was at r = 0.7 (Figure 2.3). Mean relatedness between female adult foundresses and female brood within associations was r = 0.39 ± 0.06 (range: 0.08–0.80). The highest frequency of calculated pairwise relatedness between female brood and adult foundresses within associations was at r = 0.5, with a secondary peak at r = 0.8 (Figure 2.4).

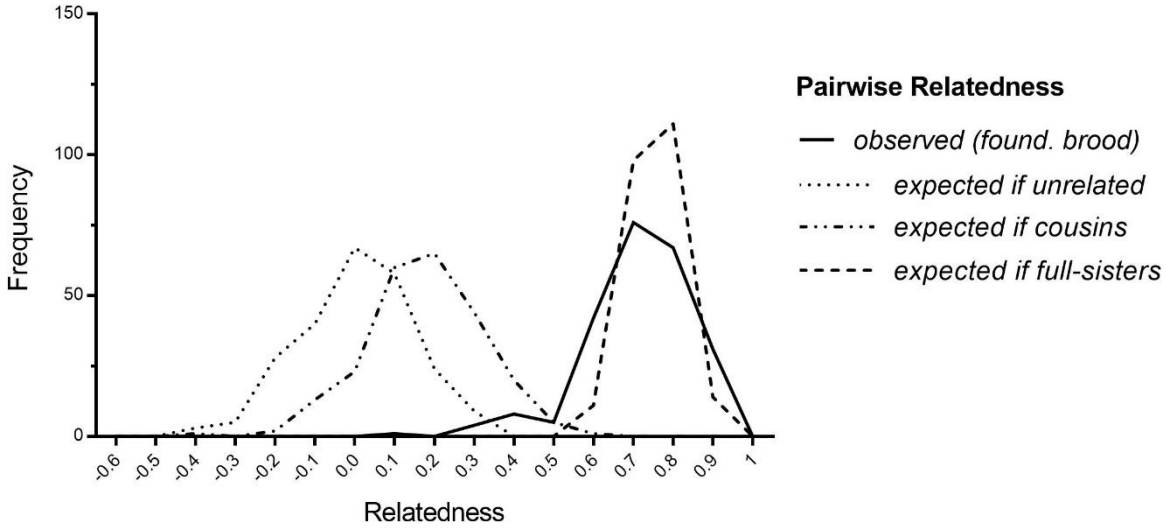


Figure 2.3. (*observed*) Pairwise relatedness distribution of female brood to each other within foundress associations; (*expected*…) simulated population (234 pairs) if the foundress brood population consisted of individuals being *unrelated* r = 0, *cousins* r = 0.1875, or *full-sisters* r = 0.75.

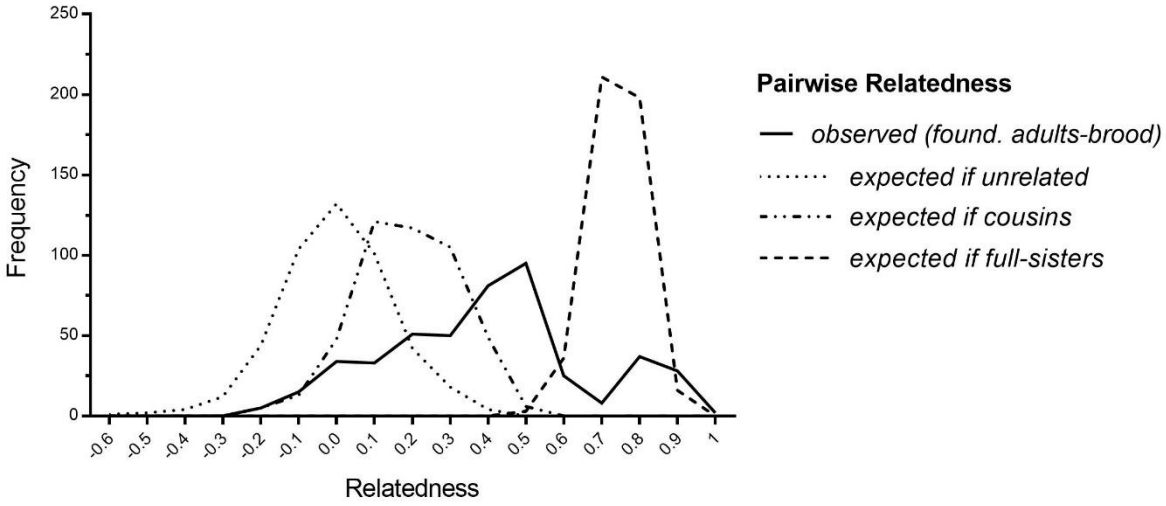


Figure 2.4. (*observed*) Pairwise relatedness distribution of adult foundresses to brood within associations; (*expected*…) simulated population (464 pairs) if the foundress adult to brood population consisted of individuals being *unrelated r* = 0, *cousins r* = 0.1875, or *full-sisters r* = 0.75.

COLONY analysis, which grouped brood into sib-groups, confirmed a reproductive monopoly in foundress associations, with a single matriline detected in eight of the ten groups analysed. A secondary egg-layer was detected in associations FA #2 and #7, and in each case this extra egg-layer contributed a single egg (Figure 2.5); in these associations, relatedness among brood was r = 0.59 and 0.76 respectively. In association FA #2, the secondary egg-layer was the mother of the other co-foundresses, with the primary egg-layer being a daughter. In association FA #7, the secondary egg-layer was collected on association FA #8, suggesting a single female was laying on multiple nests. Reproductive monopoly was further confirmed from the high reproductive skew index within foundress associations (B index = 0.651 ± 0.038, α = 0.05, range: 0.438–0.802).

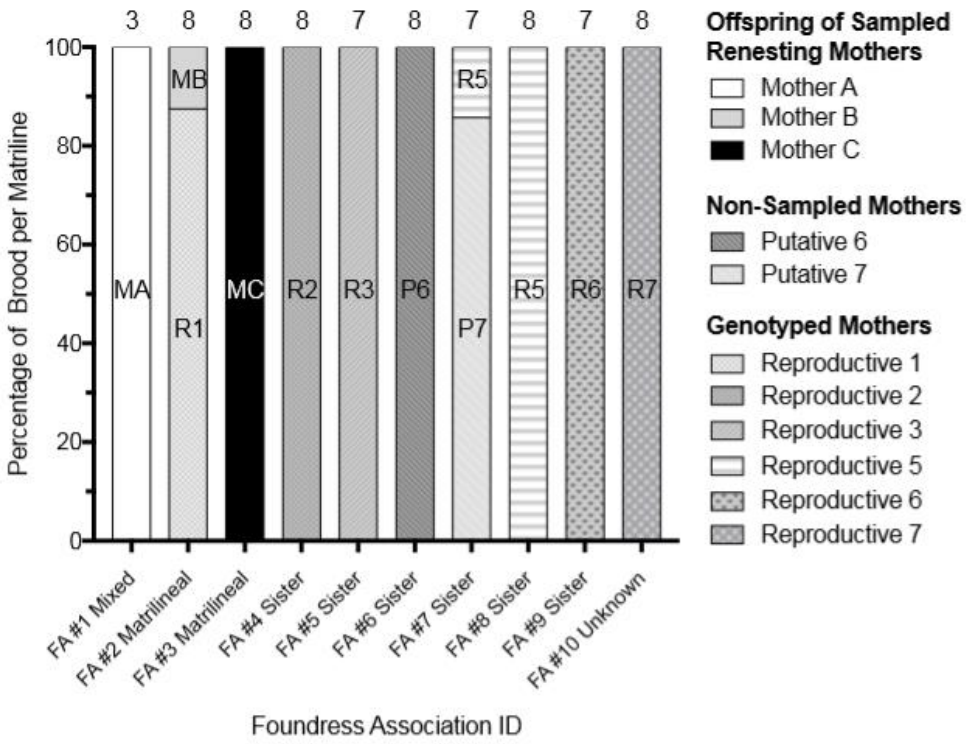


Figure 2.5. Percentage of brood (all female) in foundress associations assigned to matrilines. Three types of genetic structure were identified: *Sister* (7 associations), *Matrilineal* (2 associations), *Mixed* (1 association), and *Unknown* (1 association). Number of brood total in an association listed apex to columns. Legend denotes mother background.

Despite realized reproductive monopoly among brood in foundress associations, a mean of 46 ± 9% of adult females per association had mature egg/s present in their ovary tract. Ovarian development was most prevalent in associations with genetic structures that would yield the lowest indirect fitness for non-reproductives (i.e. *Mixed*, where 80% of females had mature eggs), whilst ovarian development was less widespread among females in associations that yielded higher indirect fitness payoffs (i.e. *Matrilineal* (58 ± 8%) and *Sister* (40 ± 13%) associations). All ten females who matched the genotypes of the predicted egg-layers had ovaries with mature eggs, scoring C (13 ± 13%) or D grade (88 ± 13%). Adult co-foundresses who were not assigned as mothers (i.e. non-reproductive co-foundresses) exhibited a range of ovary grades from A to D (A = 44 ± 9%; B = 24 ± 6%; C = 30 ± 10%; D = 3 ± 3%) (Figure 2.6). No regressed (grade E) ovaries were discovered in any co-foundress. Foundress associations did not differ significantly in the number of non-reproductive females that had mature eggs (2.0 ± 0.5) or not (3.6 ± 0.9; paired-sample t-test: $t_9 = -1.419$, $p = 0.190$) (Figure 2.7).

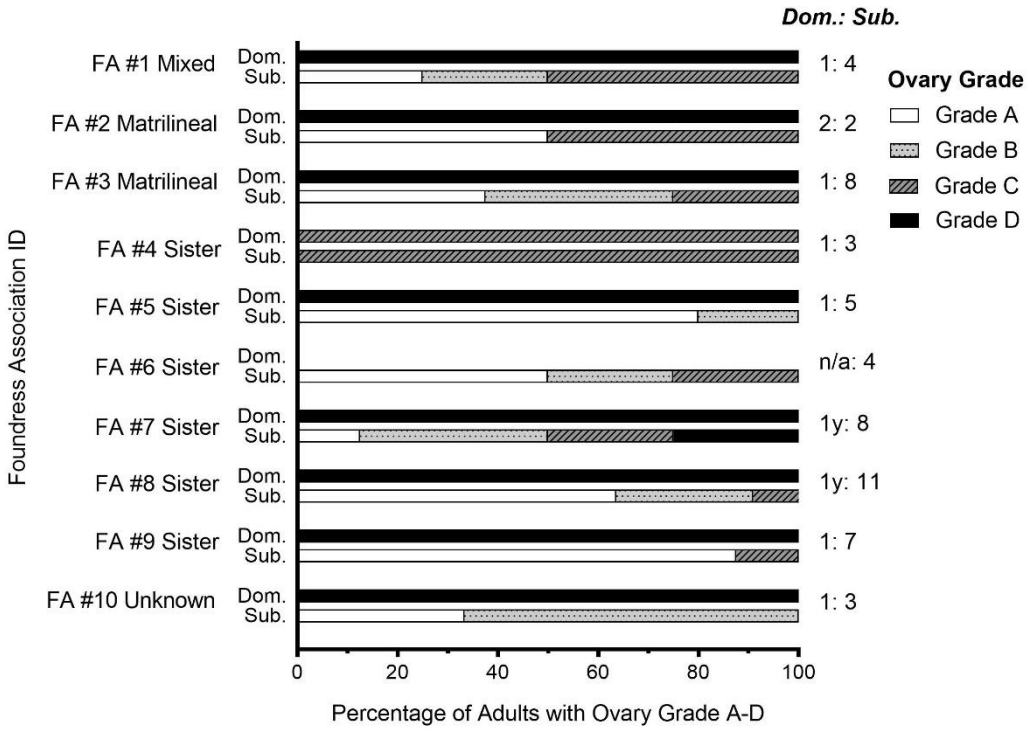


Figure 2.6. Percentage of female adults in foundress associations with ovaries graded: A = small filamentous ovarioles lacking oocytes; B = small ovarioles with slightly developed oocytes; C = large ovarioles with few developed oocytes at the base of tract; D = large ovaries with multiple fully developed oocytes; and E = large ovaries with visible regression (adapted from Gobbi et al., 2006). No grade E (large ovaries showing visible regression) were discovered. Ratio's right of columns notes the number of reproductive dominants to subordinates in the association, "y" indicates a drifter and is recorded as a duplicate across relevant associations.

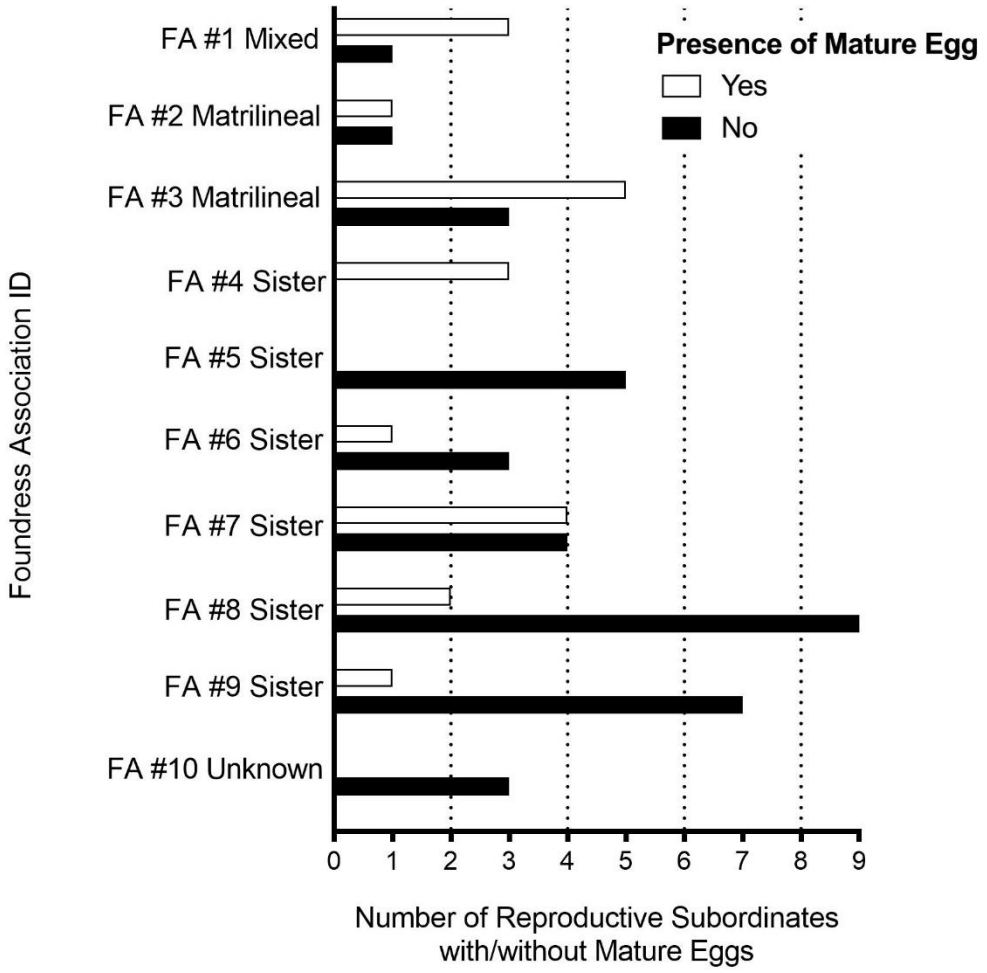


Figure 2.7. Frequency of female reproductive subordinates (i.e. had no genotyped brood found in nests) with/without a mature egg (over 2 mm in length (Sumner et al., 2006) in the ovary tract (in foundress associations).

Multiple mating (of up to two patrilines) was detected in four out of seven adult female matrilines in foundress associations (excluding four matrilines that were only represented by a single adult); k_{e3} = 1.50 ± 0.20 across groups. In nine foundress associations, mating frequency was significantly different from both k_e =1 and k_e =2, and may be a mix of single (one-sample t-test: t_6 = 2.463, p = 0.049) and twice mated (t_6 = -2.480, p = 0.048). No multiple mating was detected amongst genotyped brood of foundress associations, k_{e3} = 1.00 ± 0.00 (ten matrilines, excluding one matriline that was only represented by a single egg). This suggests that *P. canadensis* females occasionally mate more than once.

2.4.5. Hypothesis 3: Reproductive monopoly is maintained in post-emergence nests – indirect fitness payoffs for future helpers

A single egg-layer was detected in nine out of ten post-emergence nests, suggesting that high skew is maintained in these nests. In post-emergence nests, relatedness among female brood was $r = 0.73 \pm 0.01$ (range: 0.63–0.82; Table 2.3). The highest frequency of calculated pairwise relatedness between female brood within post-emergence nests was at $r = 0.8$ (Figure 2.8).

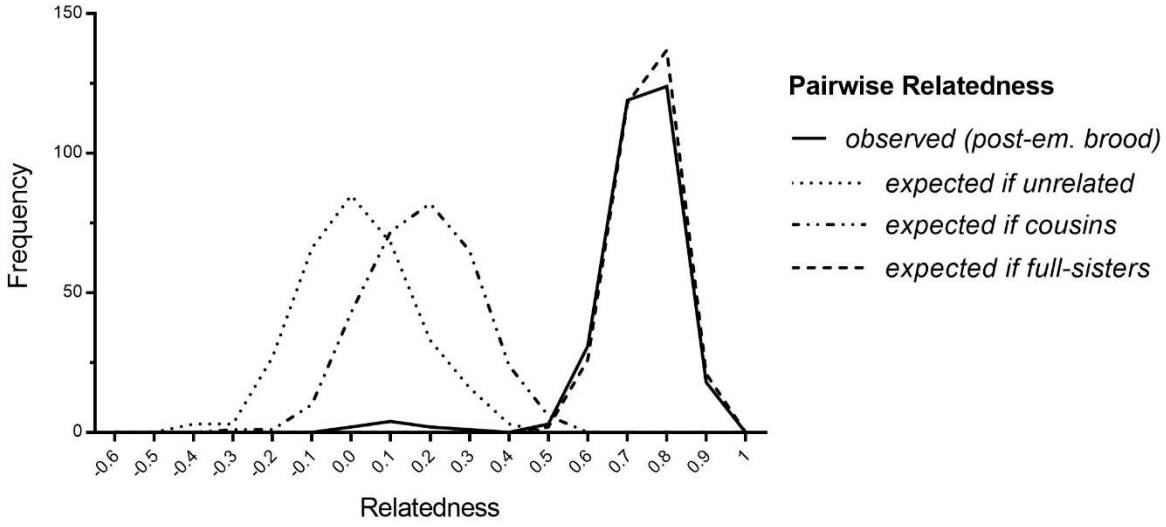


Figure 2.8. (*observed*) Pairwise relatedness distribution of female brood to each other within post-emergence nests; (*expected*…) simulated population (304 pairs) if the foundress brood population consisted of individuals being *unrelated* $r = 0$, *cousins* $r = 0.1875$, or *full-sisters* $r = 0.75$.

COLONY analysis, which grouped brood into sib-groups, confirmed a reproductive monopoly in post-emergence nests. Probability of assignment for male and female brood was 0.95 ± 0.03. The single genotyped adult forager was identified as the daughter of the dominant reproductive ($r = 0.52 \pm 0.02$) in each of the ten post-emergence nests and this individual was included as 'brood' in matrilineal analysis. Female forager-brood were assigned to a single matriline in nine nests, with one nest having a single female pupa assigned to a second matriline (Figure 2.9). Male brood were attributed to a single matriline in all ten nests (Figure 2.10). The predicted genotype of the mother of the male eggs matched that of the mother of the largest (or sole) matrilines in the female brood. The predicted mother's genotype matched that of the queen identified from behavioural observations in all ten nests. This suggests that there is almost always a single egg-layer who monopolises production of both male and female brood in post-emergence nests.

High reproductive skew across male and female brood confirmed the overall reproductive monopoly in all post-emergence nests (B index = 0.875 ± 0.021, α = 0.05, range: 0.682–0.922).

The queens identified from behavioural observations in the post-emergence nests all had D grade ovaries containing mature eggs (ten genotyped mothers). By contrast, only one forager (out of ten) had a mature egg in her ovary tract, with the most common ovary grade in foragers being A (A = 80 ± 13%; B = 10 ± 10%; C = 10 ± 10%; D = 0%). Regressed (grade E ovaries) were not found in either reproductive dominants or foragers. This suggests that secondary egglaying by daughters is rare (Figure 2.9 & 2.10), and thus philopatric females do not gain from ''sneak' egglaying. Delayed direct fitness via nest inheritance is likely the only source of direct fitness for philopatric helpers.

No multiple mating was detected in matrilines of the post-emergence nests, k_{e3} = 1.00 ± 0.00 (ten matrilines, excluding one matriline that was only represented by single brood).

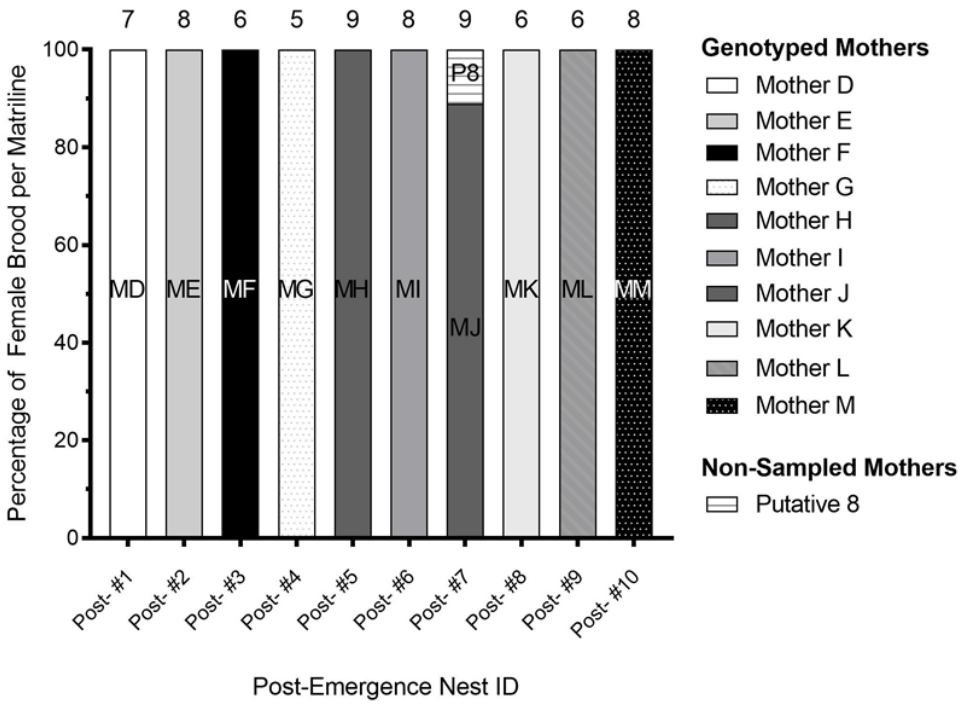


Figure 2.9. Percentage of female brood (including forager) in post-emergence nests assigned to matrilines. Number of female brood total in a nest listed apex to columns. Legend denotes mother background.

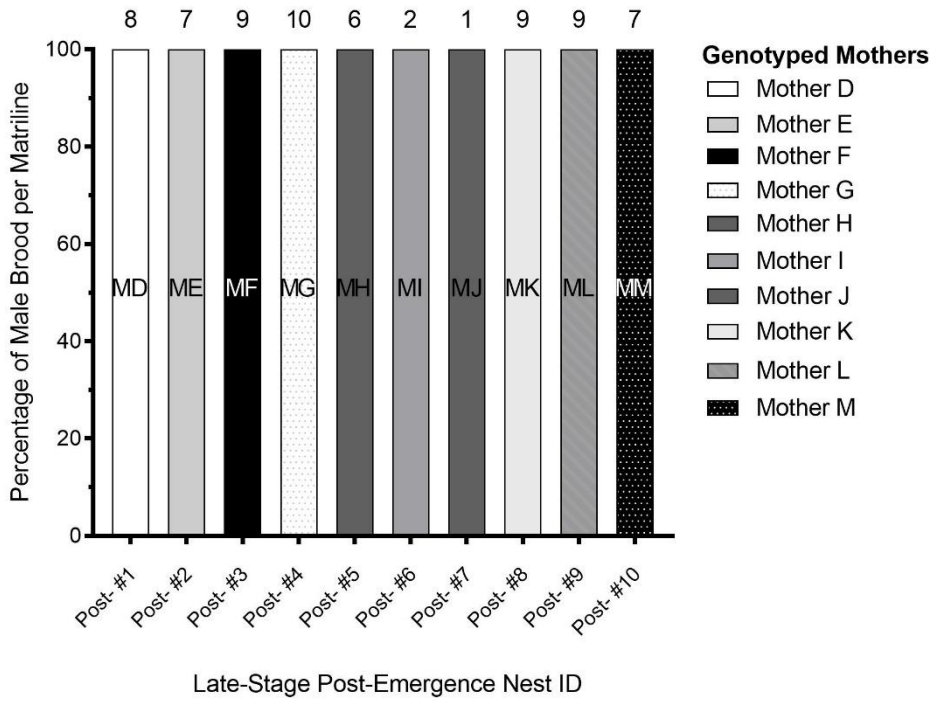


Figure 2.10. Percentage of male brood (including forager) in post-emergence nests assigned to matrilines. Number of female brood total in a nest listed apex to columns. Legend denotes mother background.

There was no evidence that male brood were derived from secondary matrilines, and thus may gain less from helping raise other brood than females do when they emerge. Potential future helpers in female and male offspring may gain indirect fitness (from maximum relatedness achievable to siblings. assuming equal sex ratios, $r = 0.5$). Because male and female brood are usually descended from the same single matriline, potential payoffs from helping are equal for males and females. The pairwise relatedness of female and male offspring to other brood on the nest was calculated using PolyRelatedness: the highest frequency of relatedness between females to other female brood was $r = 0.7$, to male brood $r = 0.2$ (simulated life-for-life $r = 0.1$), and both sexes (assuming equal sex ratios) $r = 0.5$ (simulated life-for-life $r = 0.5$) (Figure 2.11); the highest frequency of relatedness between males to female brood $r = 0.2$ (simulated life-for-life $r = 0.4$, secondary peak $r = 0.6$), to other male brood $r = 0.5$, and both sexes (assuming equal sex ratios) $r = 0.4$ (simulated life-for-life $r = 0.5$) (Figure 2.12). This is consistent with there being a single egglayer

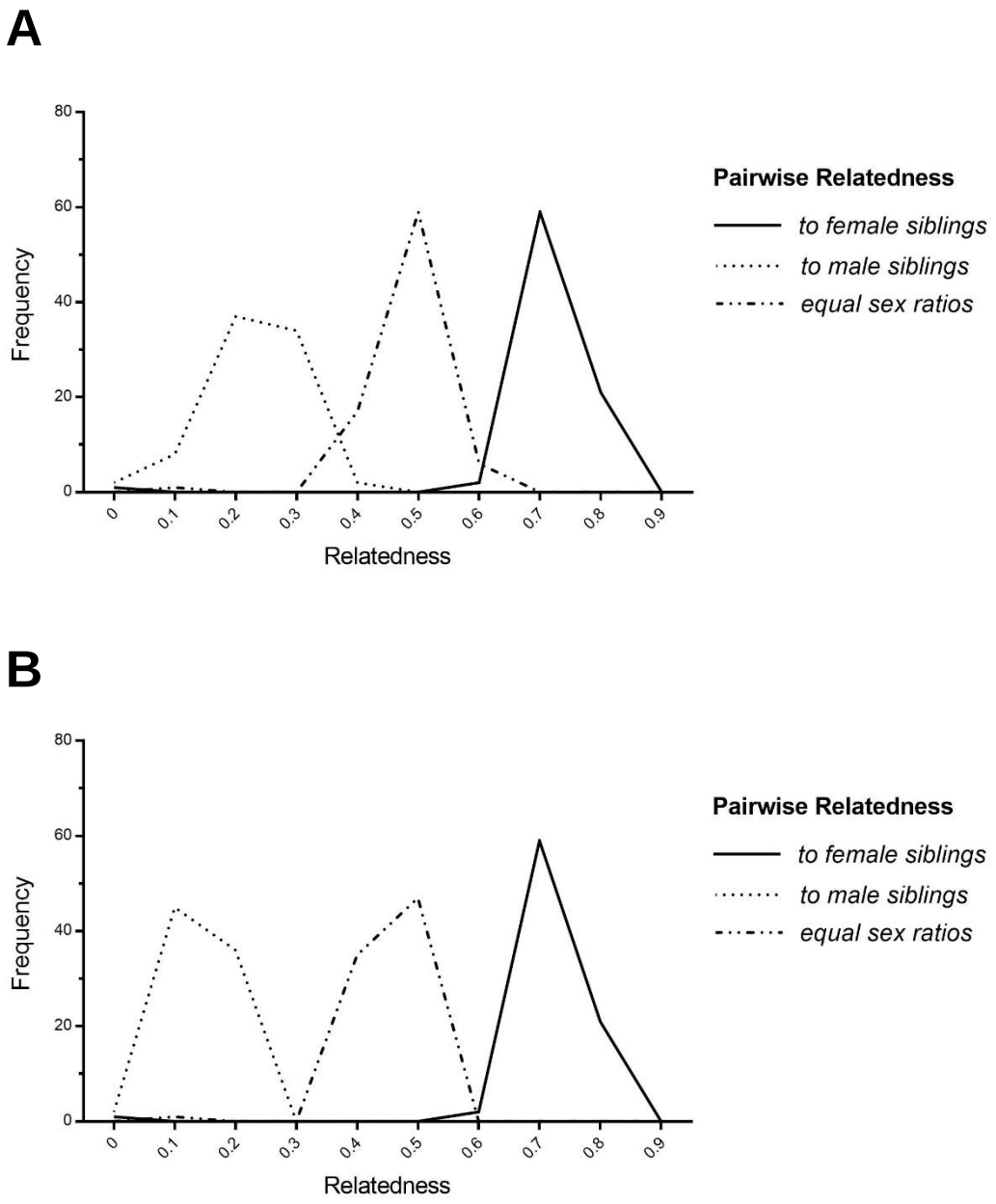


Figure 2.11. Pairwise regression (**A)** and simulated life-for-life (**B)** relatedness (Huang et al., 2014) distribution of female brood to other female and male siblings within post-emergence nests, with relatedness distribution if equal sex ratios are presumed.

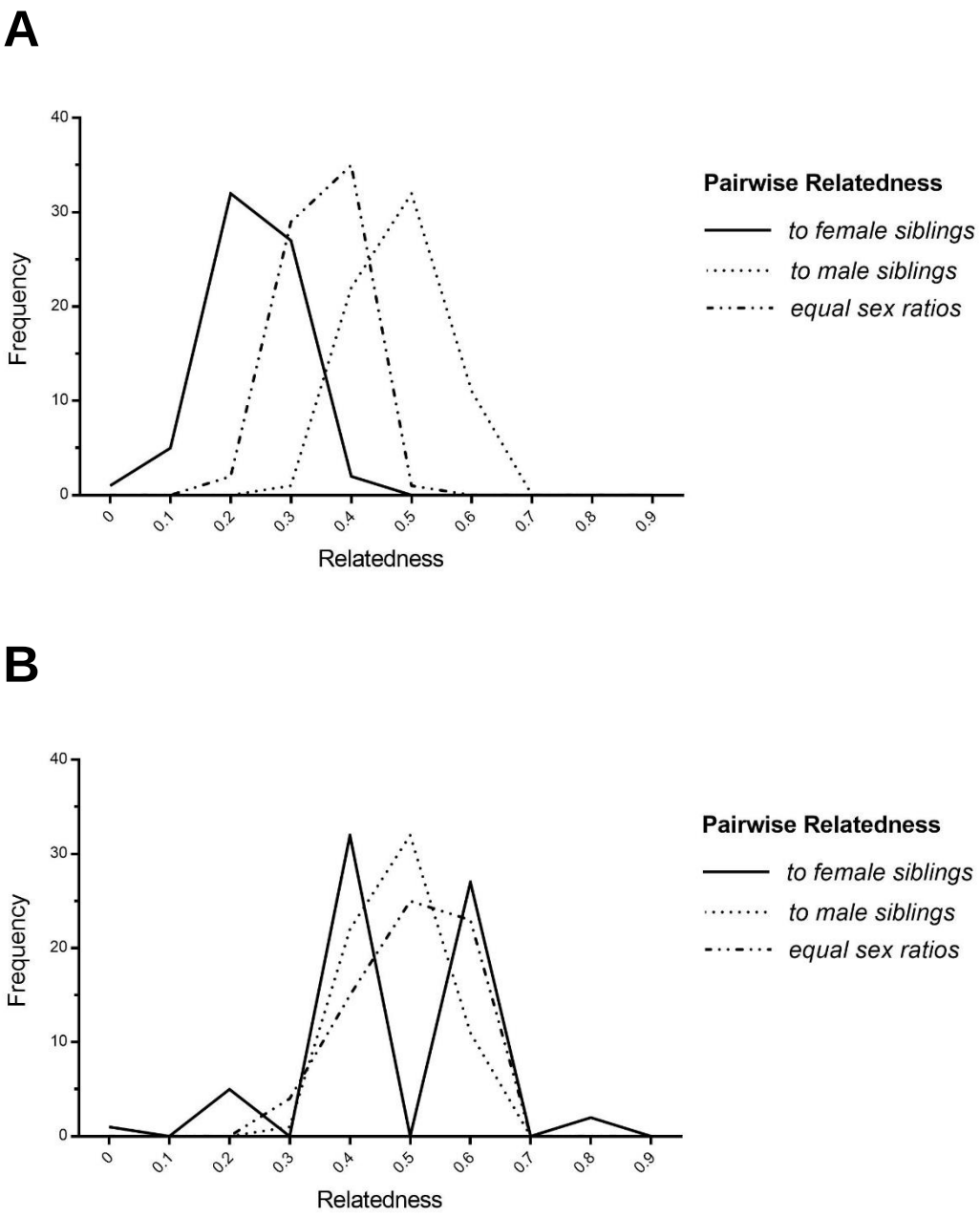


Figure 2.12. Pairwise regression (**A)** and simulated life-for-life (**B)** relatedness (Huang et al., 2014) distribution of male brood to female and other male siblings within post-emergence nests, with relatedness distribution if equal sex ratios are presumed.

2.4.6. Confirming SNP results with previous microsatellite data

Lengronne et al., (2012) reported high mean (± se) within nest relatedness of r = 0.69 ± 0.02 (Queller & Goodnight (1989) relatedness estimate) in post-emergence nests. Maternity/paternity assignment across post-emergence nests indicated that reproduction was largely monopolised. Assignment was successful in 21 of 24 of these nests (three nests were excluded from analysis with probability clustering < 0.80). Probability of assignment for female brood = 0.96 ± 0.01. A single matriline was detected amongst female brood in 85.71% (18 out of 21) of nests. Two matrilines were detected in the remaining three post-emergence nests.

High reproductive skew and a reproductive monopoly was detected in all but one post-emergence nest (B index = 0.684 ± 0.036, α = 0.05, range: 0.206–0.779). In the single nest without a confirmed reproductive monopoly (B index = 0.206), there were two matrilines split between two and three female pupae. Multiple mating (with up to three patrilines per matriline) was detected in six out of 22 matrilines in brood, k_{e3} = 1.32 ± 0.12 (excluding two matrilines that were only represented by a single brood). This was significantly different across nests from mating with either single and two mates, and so could be interpreted as being an approximate mix of single (one sample-test: t_{21} = 2.700, p = 0.013) and twice mated (t_{21} = -5.652, p < 0.001) females.

Reproductive skew of microsatellite sampled nests was significantly lower than SNP sampled post-emergence nests (t_{30} = 24.852, p < 0.001). Additionally, there was multiple mating in microsatellite sampled post-emergence nests, and not in SNP post-emergence nests. However, given that the B index was still significantly skewed in all but one microsatellite nest, and multiple mating was not confined to one dataset (i.e. found in SNP foundress associations and microsatellite post-emergence nests), it is unlikely that there were unusual anomalies in SNP genotyping.

2.4.7. Combined mating frequency

Effective mating frequency across all 48 matrilines in foundress, SNP, and microsatellite sampled post-emergence nests, k_{e3} = 1.22 ± 0.07 (excluding 7 matrilines that were only represented by single offspring). This was significantly different from a value of 1 and 2, being between single (one sample-test: t_{46} = 3.255, p = 0.002) and twice mated (t_{46} = -11.669, p <0.001). It could be concluded that *P. canadensis* do commonly mate more than once, but not enough to describe them as being multiple mated (Appendix III).

2.5. Discussion

Here we have presented the first comprehensive genetic-structure analysis of a tropical Polistine wasp, in addition to the first (to our knowledge) use of SNP based genetic markers in a social wasp (Whitfield et al., 2006; Bessoltane et al., 2012; Wang et al., 2013; Galbraith et al., 2016). Our data suggest that relatedness among foundresses is uniformly high (supporting Hypothesis 1); females generally found new nests as a mix of *Matrilineal* and/or *Sister* associations. We also show that reproductive monopoly is common at the foundress stage (Hypothesis 2), and is maintained in post-emergence nests with the reproduction of both female and male brood (Hypothesis 3). The potential payoffs from indirect fitness for totipotent non-reproductives are therefore high throughout the nesting cycle. Our findings contrast with some temperate *Polistes* species (such as *P. dominula* – Queller et al., 2000; Leadbeater et al., 2011; Field & Leadbeater, 2016), in which co-foundresses are often unrelated and thus direct reproduction is thought to be an important component of fitness. Taken together, our study posits that direct fitness may be less important than indirect fitness under certain conditions at all stages in the nesting cycle of the tropical *P. canadensis*, suggesting that life history and ecology may be important in the evolution of reproductive strategies.

High relatedness between co-foundresses in *P. canadensis* offers high indirect fitness incentives to subordinate individuals who do not reproduce. Co-founders are likely to be either raising full-sisters in *Sister* associations (r = 0.375) or female siblings in *Matrilineal* associations (r = 0.75). Since lone foundresses in *Polistes* species have a very low survival rate and low productivity compared to nests containing multiple co-foundresses (West-Eberhard, 1969; Pickering, 1980; Reeve, 1991; Tibbetts & Reeve, 2003), the payoffs to helping are likely greater than attempting to reproduce alone. As observed by Pickering (1980), new foundress associations in *P. canadensis* may also be continuations from previously abandoned mature post-emergence nests, which we have shown these associations often consist of close relatives (e.g. a mother with some daughters). In the absence of an overwintering diapause period, opportunities to nest with relatives may be more prevalent for tropical species than for temperate social wasps, as the time lag between leaving the natal nest and founding a new nest is likely short in tropical species. There is weak evidence of precise kin recognition in terms on relatedness within nests of temperate *Polistes*, although wasps may recognise nestmates or discrete units of kin (i.e. related vs. unrelated) (Gamboa, 2004; Leadbeater et al., 2013). Co-founding individuals may be making joining decisions based on advertised signals such as behaviour and nestmate odour, or follow simple rules such as distance from the natal nest (Field & Leadbeater, 2016).

Reproductive monopoly was evident at both stages of the nesting cycle. High skew at the founding stage generates conditions for altruism to evolve through indirect fitness benefits.

But for helpers to realise these fitness benefits, high skew must be maintained throughout the nesting cycle, to the point where new reproductive (gynes and males) are produced. Our findings contrast those from some temperate *Polistes* species, where lower reproductive skew (more egg-layers) is often found in the first brood and skew then increases as the nesting cycle progresses, such that the reproductive brood (gynes and males) are the offspring of a single original foundress (Field et al., 1998; Reeve et al., 2000; Seppä et al., 2002). Early skew in *P. canadensis* is alike to the genetic-structure found in other non-swarm founding tropical wasps, such as *Parischnogaster alternata* (Bolton et al., 2006) and *Liostenogaster flavolineata* (Sumner et al., 2002). Regarding male production, we found no evidence of male egg-laying by secondary egg-layers (Miyano, 1983; Arévalo et al., 1998; Strassmann et al., 2003). In calculating female and male relatedness to other post-emergence siblings, if equal sex ratios are presumed then both males and females are related by $r = 0.5$ to other brood. Therefore, if both sexes had equal costs for helping raise siblings, then indirect fitness benefits would also be equal. However, because males may achieve $r = 1.0$ to producing daughters, dispersal and mating is likely always the optimal fitness payoff – unless mating was temporary unachievable (i.e. from delayed maturation or limited mating opportunities in the environment, see Chapter 5).

An interesting question raised by our findings is how reproductive monopoly can be maintained in such large nests. Our nests spanned up to 80 females; but nests can become much larger, with groups of 100+ wasps observed commonly (Pickering, 1980; Hunt, 2007; authors pers. obs.). Dominance hierarchies in *Polistes* are established and maintained primarily through physical aggression and threat displays, such that suppression of reproduction by subordinates is achieve by queen control. In *Polistes*, reproductive dominance is enforced through physical aggression of subordinates (Jandt et al., 2014). *Polistes* queens in large tropical nests are unlikely to be able to dominate all females on the nest physically; it was suggested that such queens would be forced to concede reproduction to additional egg-layers in certain 'territories' of the nest (West-Eberhard, 1986). Our analyses indicated that if territorial queens did exist in post-emergence nests, they did not have large effect on overall reproductive skew.

Despite mostly monopolised reproduction of brood in foundress associations, various levels of ovarian development existed across reproductive subordinates, with 46% possessing mature eggs in the ovary tract. This differed from post-emergence nests, where only one in ten subordinate individuals sampled had a mature egg – in line with the findings of Sumner et al. (2010), reporting only 3.6% of *P. canadensis* helpers possessed mature eggs. In foundress associations, sisters may be 'queueing' to reproduce should the dominant egg-layer disappear, this would mean a component of direct fitness incentives still exist in tropical species (as in temperate – Leadbeater et al., 2011; Field & Leadbeater, 2016). Given that additional matrilines on foundress and post-emergence nests were rare, nest inheritance may be the only

source of direct fitness for philopatric helpers. This is of relevance when considering any asymmetries in the payoffs of staying on the nest to help in males and females: specifically, sneaky direct fitness (i.e. egglaying without the queens notice) is not likely to be augmenting the inclusive fitness of females who remain at the natal nest and help. However, assessment of reproductive partitioning from ovarian development along is a poor representation of actual reproduction in *P. canadensis* foundress associations. Previous studies found ovarian development an unreliable indicator of reproductive partitioning (Strassmann et al., 1983; Seppä et al., 2002; Izzo et al., 2010; Cini et al., 2013).

The discovery of multiple mating in this species is surprising, since it has only been reported once previously in *Polistes* (average mating frequency in *Polistes* = 1.01, in Strassmann, 2001). The effective mating frequencies of 1.22 found in *P. canadensis* are higher than those of other social wasp species such as *Vespa cabro* (1.11), or *Dolichovespula spp.* (1.13), but much lower than highly polyandrous species such as *Apis mellifera* (12.40) (reviewed in, Strassmann, 2001). Multiple mating is a trait of complex sociality, found in species that have evolved beyond the 'monogamy window'. A defining feature of eusocial insects is that they mate during one period in their lives: it has been suggested that this is one trait that sets truly eusocial species apart from cooperative breeders (Boomsma, 2009). By this argument, *Polistes* therefore may be better described as a cooperative breeder than a eusocial organism (Boomsma, 2009). Determining whether females remate later in life is required to test this hypothesis further, as Hymenoptera are usually characterised as mating once in their life (Boomsma, 2007; Hughes et al., 2008).

High relatedness and reproductive skew are likely to be key components in indirect fitness gains for reproductive subordinate co-foundresses in the tropical *P. canadensis*, which facilitates the continuation of high relatedness in female and male brood and thus the presence of future helping nestmates. Our study raises the question of whether direct fitness via nest inheritance is an ancestral trait in the evolution of cooperation in *Polistes*, or a secondary adaptation to diapause in temperate regions. Further studies on species spanning temperate to tropical climates are therefore encouraged to fully understand the influence of environment on social structure and dynamics at the origin of group living.

2.6. Appendix List

Associated appendixes: Appendix I – genotyping sample list; Appendix II – SNP loci information; Appendix III – combined effective mating frequency.

Chapter 3. Skewed reproduction and high offspring relatedness in established nests of the Neotropical paper wasp *Polistes lanio*

Contributions: R. Southon, A. Radford, & S. Sumner designed the study; R. Southon & S. Sumner conducted the fieldwork, with help from summer intern J. Valverde; P. Graystock & C. Wyatt identified SNPs from existing transcriptome RNA-Seq assemblies; R. Southon conducted SNP validation, sample genotyping, all other lab-work, and analyses.

3.1. Abstract

The eusocial societies of Hymenoptera typically possess high reproductive skew, with a single or few breeding queens, and many effectively sterile female workers. If reproduction is monopolised in societies, then offspring will have a higher average relatedness via shared parentage than if reproductive partitioning were more egalitarian. In the simple eusocial Polistinae paper wasps, reproduction tends to be monopolised in established post-emergence nests, despite many workers retaining the ability to mate and reproduce. Most genetic studies of reproductive partitioning in the paper wasp genus *Polistes* have been limited to temperate species. Tropical *Polistes* species are understudied, and experience differing environmental pressures to temperate species, which may result in contrasting reproductive contracts among totipotent nestmates. Previously, we found in nests of the tropical *Polistes canadensis* that high reproductive skew and high relatedness among offspring was maintained throughout the nesting cycle (Chapter 2). We predict that skewed reproduction is a common trait among tropical *Polistes* species. Here we examine the genetic structure of nesting groups in the tropical wasp *Polistes lanio*, a sister species of *P. canadensis*, using single-nucleotide polymorphism (SNP) loci to assign brood to mothers and sib-groupings. We found that reproductive skew is monopolised by a single reproductive queen, and that relatedness between offspring (both female and male pupae) is high. Additionally, we found evidence of queen turnover in two of the ten genotyped nesting groups, whereby the collected queen was the sister of the pupae brood. A reproductive monopoly and subsequent high relatedness between offspring is likely to be typical of tropical *Polistes.* Furthermore, we may expect to find behaviours in post-emergence nests of *P. lanio* that capitalise on potential indirect fitness gains between siblings with shared parentage – such as male helping (Chapter 6).

3.2. Introduction

Social groups that are composed of many potential breeders must resolve within-group conflict over who reproduces (West et al., 2002; Clutton-Brock, 2009). Reproductive skew theory describes the outcome of such reproductive conflict, in the form of how breeding is partitioned among potential reproductives within groups. High reproductive skew is depicted in groups where reproduction is monopolised by a few breeding individuals, whilst low skew can be found in egalitarian societies in which reproduction is equally shared (Reeve & Keller, 2001; Ratnieks et al., 2006). Determining genetic relationships between breeders, nonbreeding helpers, and juvenile offspring allows quantification of reproductive skew, and provides an overview of the potential ultimate fitness payoffs of behavioural actions that may promote indirect or direct fitness (Hamilton, 1964; Trivers, 1971; Queller & Goodnight, 1989; Queller, 1992). Revealing the social contracts between group members advances our understanding on the evolution and maintenance of sociality. Here we analyse genetic structure data to estimate within-group relatedness and parentage of offspring in nests of a simple eusocial paper wasp.

The eusocial hymenopteran societies typically possess high reproductive skew, with a few reproductive egglaying queens and many related female workers that are either functionally or effectively sterile (Wilson, 1971). The simple eusocial hymenopteran societies represent the early stages of eusociality, in which many females in small nesting groups retain the ability to reproduce (Danforth & Eickwort, 1997; Bell & Sumner, 2013). Determining how conflicts over reproduction are resolved in these simple eusocial societies provides key insights into the evolution of eusociality and reproductive division of labour (West-Eberhard, 1969; Reeve, 1991).

The *Polistes* paper wasps are a cosmopolitan genus, with simple eusocial societies consisting of relatively small groups (compared to complex eusocial wasp societies), in which all nestmates emerge as adults with the ability to reproduce (Pardi, 1948; West-Eberhard, 1969; West-Eberhard, 1996; Bell & Sumner, 2013; Jandt et al., 2014). Despite being geographically widespread, most studies have focused on temperate species, particularly studies which examine genetic structure (Queller et al., 1997; Field et al., 1998; Reeve et al., 2000; Seppä et al., 2002; Liebert et al., 2005; Leadbeater et al., 2011). The *Polistes* genus likely has tropical ancestry (Carpenter, 1996; Santos et al., 2015), and there are key differences in the ecologies of temperate and tropical species, which may alter reproductive opportunities for breeders and helpers. In contrast to temperate *Polistes* species, tropical species are essentially perennial, lacking the termination of the nesting cycle for a period winter diapause. The tropical *Polistes* species are also characterised by the emergence of adult females and males throughout the year in the population; moreover, nests become much larger in the tropics than their temperate counterparts, both in terms of group size and cell number (West-Eberhard,

1969; Pickering, 1980; Judd, 1998; O'Donnell & Joyce, 2001). Because of these ecological and life history differences, we cannot assume that the social, breeding and mating structure of tropical *Polistes* is the same as that of temperate *Polistes.* We lack fine-scale genetic analyses of family structures and reproductive skew among group members in tropical *Polistes* species, as previously highlighted in our *Polistes canadensis* study (Chapter 2). Genetic structure data is essential for a proper understanding of potential variation in social organisation, and in how conflicts over reproduction are resolved at the origin of sociality in this cosmopolitan genus (Bourke, 2014).

Genetic markers allow family structures to be revealed; for example, assignment of parentage to individual offspring provides a measure of reproductive skew and a snap-shot into the possible resolution of conflicts in societies (Queller & Goodnight, 1989; Smith et al., 2008). Allozymes were the first genetic markers to be used to address genetic relationships in animals, but suffered from a restricted sampling methodology and limited number of variable markers available (Murphy et al., 1996; Schlötterer, 2004). In the 1990s, PCR amplification of microsatellite markers (di-, tri-, or tetra- base repeat sequences) revolutionised the capacity to discern fine-scale genetic relationships within and among populations. However, microsatellites also suffer limitations, from small numbers of marker, unknown distributions through the genome, high mutation rate, and a difficultly in utilisation for cross-species comparisons (Ellegren, 2004; Schlötterer, 2004; Putman & Carbone, 2014). Single-nucleotide polymorphism (SNP) markers provide a high-resolution alternative to microsatellites, from large sets of markers selected across the genome that are easily identified using transcriptome data, making SNPs ideal for kinship analysis (Vignal et al., 2002; Putman & Carbone, 2014). In this study, we use SNP generated genetic structure data to examine relatedness and reproductive partitioning in the Neotropical paper wasp *Polistes lanio*, a species with a distribution across the South American continent (Carpenter, 1996).

Our study has three aims. First (Aim 1), to develop and validate a set of SNP markers for *P. lanio* that has potential cross-species use with the sister species *P. canadensis*. Second (Aim 2), to use SNP markers to determine shared parentage and relatedness between selected adults and brood within nesting groups. We predict that there will be high female reproductive skew among nestmates in established post-emergence nests of *P. lanio*, indicating that a single female monopolises reproduction. Finally (Aim 3), we analysed whether females are multiply mated, since data in the sister species *P. canadensis* indicated some females mate twice (Chapter 2).

3.3. Methods

3.3.1. Sample collections

Ten established post-emergence nests of *P. lanio* were collected during the wet season in Trinidad, Trinidad & Tobago (two sites of approx. two hectares each). In June 2013, five nests were collected from Verdant Vale (VV), Blanchisseuse Rd, 10°41'5.44"N, 61°17'24.95"W, and in August 2014 five nests were collected from both Verdant Vale (VV) (same coordinates) and Eastern Main Rd (EM) 10°39'1.21"N, 61°15'9.63"W. Post-emergence nests were defined as having evidence of emerged natal adults (broken pupae caps), indicating that some adult offspring had eclosed and thus the nesting group was established with new workers. All nests had a single comb. Prior to collection, two female phenotypes were identified in sampled nesting groups. A queen was identified by removal of an egg from a cell, and observing which individual subsequently oviposited within an hour (West-Eberhard, 1986). On the five nests collected in 2013, a forager was identified as a female who returned to the nest with solid forage material in her mandibles. On the five nests collected in 2014, all wasps were individually marked using extra fine tip Uni POSCA markers, and censuses of nest membership were taken at least once a day during daylight hours for 5–22 days. Female foragers were identified as wasps that had been present the least number of censuses, being observed on the nest a mean 50% of censuses. Collections of whole nests with adults and brood were conducted at dusk, when most foraging wasps are likely to have returned to the nest to spend the night. Collected samples were stored in 80% ethanol at -20°C. The number of cells and brood in the nest was counted upon collection, and each cell categorized as having an egg, larva, pupa, or being empty or parasitised. Nesting groups consisted of a mean 12.3 ± 2.0 number of adult females, with combs of 111.3 ± 27.6 cells (Table 3.1). To represent a nesting group, a random sample of five pupae with the two identified adult females (queen and forager) were chosen for genotyping analysis.

3.3.2. Aim 1: SNP-loci discovery and validation

SNPs were identified from transcriptome RNA-seq assemblies for ten individuals of *P. canadensis* (collected from Panama - Patalano et al., 2015) and ten individuals of *P. lanio* (from Verdant Vale (VV), Trinidad - Moreno et al., in prep.). To validate a first set of SNPs, 35 *P. lanio* pupae (5 pupae each from 7 nests) were genotyped at 120 loci using KASP™ SNP genotyping (methods for DNA extractions and KASP assays as described in Chapter 2). Polymorphic loci were selected by manually examining clusters, removing monomorphic loci, those with unclear allelic clustering, and loci with Minor Allele Frequencies (MAFs) < 5%. This left 93 loci used for genotyping all remaining samples across the ten nests to be analysed in our genetic structure study. Loci validation was further refined by testing and removing those loci that deviated from Hardy-Weinberg equilibrium (HWE) (Lange et al., 2005). We report levels of linkage disequilibrium (LD) using Fisher's exact test between each pair of loci (Lazzeroni & Lange, 1998). Tests for HWE and LD were performed in the software package Mendel 14.4

(Lange et al., 2013). A final set of 86 SNP loci were used in all subsequent analyses of genetic structure (with 59 loci viable for use in both sampled *P. canadensis* and *P. lanio* populations, see Chapter 2, Appendix II). All samples were genotyped at a minimum of 67 loci, with 35 samples genotyped at 86 loci.

Table 3.1. Characteristics of collected and genotyped post-emergence nests of *P. lanio*. Sites used across the thesis: VV = Verdant Vale; EM = Eastern Main Rd.

Nest ID	Year	Site	Nest Characteristics								
			Number of Adult Females	Number of non-Adult Offspring	Total Number of Cells	Number of Eggs	Number of Larvae	Number of Pupae	Number of Empty Cells	Number of Parasitised Cells	Ratio of Adults to Larvae
#01	2013	VV	9	51	52	21	15	15	0	1	0.60
#02	2013	VV	6	65	65	27	29	9	0	0	0.21
#03	2013	VV	10	95	95	37	36	22	0	0	0.28
#04	2013	VV	6	53	53	21	19	13	0	0	0.32
#05	2013	VV	5	41	41	16	20	5	0	0	0.25
#06	2014	VV	8	90	92	42	36	10	2	0	0.22
#07	2014	VV	18	96	96	47	32	17	0	0	0.56
#08	2014	VV	20	104	126	35	33	14	22	0	0.61
#09	2014	EM	21	191	338	22	13	9	147	0	1.62
#10	2014	EM	20	123	155	35	38	18	32	0	0.53
Mean ± SE			12.30 ± 2.09	90.90 ± 13.93	111.30 ± 27.57	30.30 ± 3.27	27.10 ± 2.98	13.20 ± 1.60	20.30 ± 14.53	0.10 ± 0.10	0.52 ± 0.13

3.3.3. Aim 2: Nesting group genetic-structure analyses

SNP genotyping: To determine nesting group genetic structure, five pupae and two female adults (identified as a queen and forager phenotype) from each of the ten nests were analysed. Any brood genotyped as homozygous at all loci were identified as males. No heterozygous diploid males were detected (Liebert et al., 2004). Accuracy and replicability of genotyping was checked by rerunning at least three negative (no DNA) and two positive (duplicate or repeats) controls per PCR plate (two PCR plates per loci).

Nesting group relatedness and genetic structure: Female pairwise relatedness (females only) was estimated using COANCESTRY 1.0.1.5 (Wang, 2011), reporting the Wang (2002) relatedness estimation. Mean relatedness across groups was calculated using the mean of each group, so each group had equal weighing irrespective of the number of individuals sampled (Lengronne et al., 2012). Pairwise relatedness of individuals within groups was plotted against simulated populations consisting of unrelated (r = 0), cousin (r = 0.1875), and full-sister (r = 0.75) pairs (pairwise relatedness to 1 d.p.). Simulated populations were created with KINGROUP v2_090227 (Konovalov et al., 2004), based on the observed allele frequencies of females in the genotyped population (using the Queller & Goodnight relatedness estimation (Queller & Goodnight, 1989)). Individual F_{IS} inbreeding coefficients of females in nests were derived from the Lynch & Ritland (1999) estimate calculated in COANCESTRY, and tested for significant differences from a coefficient of zero (no significant inbreeding) using one-sample t-tests for the two stages of the nesting cycle (foundress and post-emergence).

Pedigree structure: Adults and brood were assigned to sib-groups, and the genotypes of their putative mothers (matrilines) and fathers (patrilines) predicted based on allele sharing using COLONY 2.0.6.3 (Jones & Wang, 2010). A full-likelihood method using all genotypes in a single model was used, and assignment of clusters accepted only when the probability of assignment was ≥0.75. We allowed for the possibility of polyandry (multiple mating by females) in matriline assignment, as analyses in Chapter 2 detected a low level of multiple mating in *P. canadensis*. Parental assignment was conducted by first including all adults as potential mothers to the pupae across all ten nests (this was relevant as wasps are known to drift between nests, Sumner et al., 2007). Second, any adults not assigned as a likely mother to brood were reanalysed as potential siblings of brood. Accuracy of assignment was judged using the sub-structure function in COLONY.

Reproductive Skew: An index for reproductive skew was calculated for each nesting group to quantitatively assess how reproduction was partitioning amongst potential reproductive females (i.e. total number of adult females on the nest). The B index was used to measure reproductive skew of matrilines, which is suitable for small sample sizes combining observed and expected variances (Nonacs, 2000), whereby values closer to 1 indicate higher skew

(Nonacs, 2003). The B index was calculated using SKEW CALCULATOR 2003 (© Peter Nonacs), with 1000 simulations, 95% confidence intervals, and α = 0.05. The default setting which assumed equal length of time spent on the nest between potential mothers was used, as the actual tenure of individual adult females in the group was not known.

3.3.4. Aim 3: Multiple mating

The effective mating frequency of females k_{e3} (Nielsen et al., 2003) is reported across matrilines, excluding any matrilines consisting of a single genotyped offspring (data from COLONY analysis). Combined effective mating frequencies across matrilines were then compared to a k_{e3} of one (singly mated) and two (twice mated) using a one-sample t-test.

3.3.5. Statistical analyses

Statistics, unless otherwise stated, were performed in R 3.3.3 (R Core Team, 2017) to α = 0.05, reporting the mean ± SE (2 and 3 d.p.).

3.4. Results

3.4.1. Aim 1: SNP-loci discovery and validation

From the initial 120 SNPs used for validation, 12 loci failed manual cluster evaluation, two loci had MAFs less than 5%, and a further 13 loci had poor genotyping clarity. Upon filtering these loci, 93 loci remained. Significant deviations from HWE were detected in seven loci and removed, with LD (Fisher's exact test) detected across 88 pairs of remaining loci (2.41% of pairings). This left a final set 86 loci for use across all samples, with MAFs > 10%. Individual samples were successfully genotyped and had a mean coverage across 94.33 ± 0.55% of the 86 loci. No assignment errors between plate samples and positive controls were detected. Three male pupae were identified in pupae (1 male pupae, in 3 nests).

3.4.2. Aim 2: Nesting group genetic-structure analyses

Nesting group relatedness and genetic structure: Mean relatedness among sampled female pupae was high across nests (r = 0.73 ± 0.1, range: 0.64–0.78). The highest frequency of calculated pairwise relatedness between female pupae was at r = 0.7 (Figure 3.1). A female heterozygosity of H_{obs} = 0.441 ± 0.017 (range: 0.033–0.750) was observed across all 86 loci. No significant inbreeding or outbreeding was detected, F_{IS} = -0.024 ± 0.015 (lower/upper 95% CI: -0.054/0.006), with no significance from a F_{IS} of zero (t_{66} = -1.623, p = 0.109).

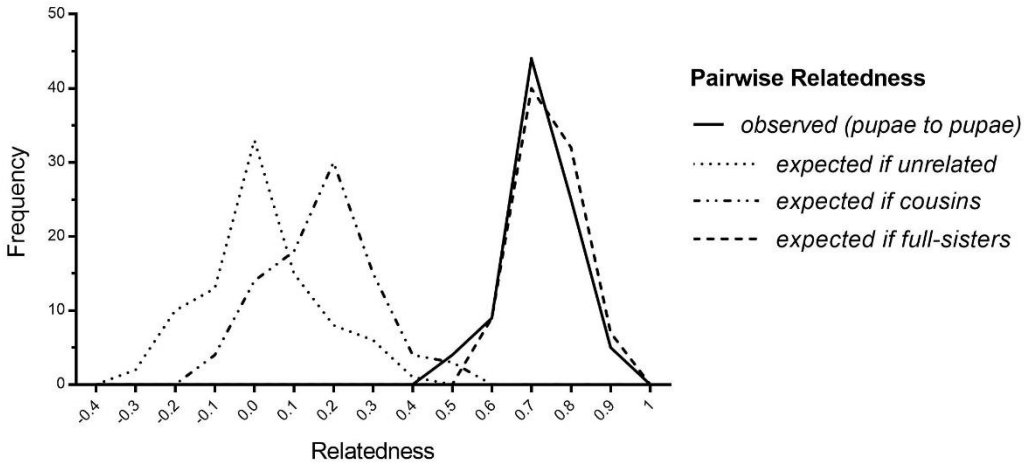


Figure 3.1. (*observed*) Pairwise relatedness distribution of female pupae to other female pupae within nests; (*expected*…) simulated population (88 pairs) if the nest consisted of individuals being *unrelated r* = 0, *cousins r* = 0.1875, or *full-sisters r* = 0.75.

Pedigree structure: COLONY analysis, which grouped samples into sib-groups, confirmed close relatedness within nesting groups. Probability of assignment in nine nests was 0.97 ± 0.01 (one nesting group was removed with a probability < 0.75). A single wasp was detected as the mother of female (and male when applicable) pupae in each nest. In seven nests, the genotype of the predicted mother matched that of the female identified as a queen from behavioural observations, with the forager also assigned as the offspring of the queen. In two nests, the behavioural egglayer (identified from behavioural observations) was not the mother to pupae; instead these females were assigned as a full-sister (nest #09) and half-sister (nest #08; shared mother, different father) to nestmate pupae respectively. In these two nests with sister egglayers, the adult foragers were assigned as a full-sister to pupae and of no relatedness to the egglayer or pupae respectively. A possible explanation for a lack of an assigned mother in these two nesting groups is that there might have been queen turnover prior to collection (Table 3.2, Figure 3.2).

Reproductive Skew: As expected from relatedness and pedigree assignment analysis, high reproductive skew in both female and male pupae was detected across nine successfully assigned nests, confirming an overall reproductive monopoly present in each nesting group (B index = 0.723 ± 0.013, α = 0.05, range: 0.667–0.764).

3.4.3. Aim 3: Multiple mating

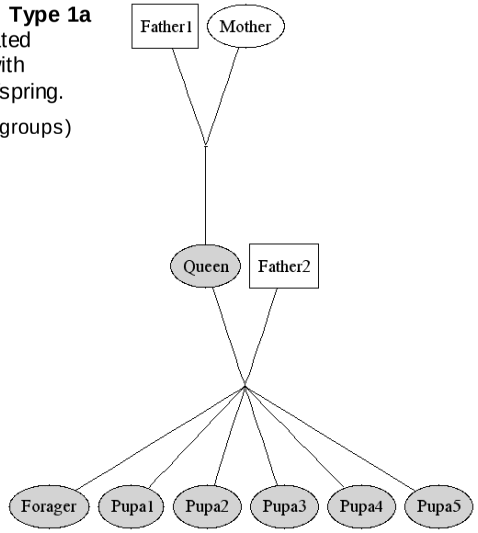
Multiple mating, with up to two patrilines per matriline, was detected in three out of nine matrilines for which multiple offspring had been genotyped, $k_{e3} = 1.15 \pm 0.08$. In each case this represented a split between the patrilines of pupae and adult offspring. Overall this was not significantly different from a $k_e = 1$ ($t_8 = 15.333$, $p = 0.081$), and was significantly different from a $k_e = 2$ ($t_8 = -11.333$, $p < 0.001$). In each of the three cases of multiple mating, pupae clustered in one patriline and adults in the other. To check that this split pattern of paternity across generations was not an artefact of the COLONY analysis procedure, we reanalysed the data such that all individuals (including egglayers and foragers) were treated as potential offspring with unknown mothers (Appendix IV). In each matriline, pupae all belonged to a single patriline, and the adults to a second or third patriline. This suggests that the patriline-specific clustering by generation is likely to be a real effect.

Table 3.2. Number of unique matrilines (with patriline partners) within each nesting group (COLONY analysis), listed with colony relatedness and reproductive skew. Legend: *Obs.* = observed queen or forager by behavioural/census assessment; ♕observed queen; ♘observed forager; ♗pupae.

Nest ID	Description of Nest Structure	Unique Matriline Description	Unique Patriline in Female Offspring 1	Unique Patriline in Female Offspring 2	Number of Male Pupae Genotyped	Relatedness of Observed Queen to Forager	Mean Relatedness within Female Pupae	Reproductive Skew in Pupae of both sexes (B index)
#01	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 5♗	 1♘	0	0.68	0.69	0.711
#02	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 5♘♗		1 ♕ offspring	0.23	0.70	0.667
#03	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 6♘♗		0	0.50	0.74	0.720
#04	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 5♗	 1♘	0	0.72	0.76	0.667
#05	*Removed <0.75 cluster probability* (1 male pupae genotyped)							
#06	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 5♘♗		1 ♕ offspring	0.46	0.75	0.700
#07	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 6♘♗		0	0.50	0.72	0.756
#08	Queen Turnover	*Obs. egglayer & pupae's non-genotyped mother* *Obs. forager's non-genotyped mother*	5♗ 1♘	1♕	0	-0.03	0.64	0.762
#09	Queen Turnover	*Obs. egglayer, forager, & pupae's non-genotyped mother*	7♕♘♗		0	0.69	0.78	0.764
#10	Mother with offspring	*Obs. egglayer's non-genotyped mother* *Obs. egglayers offspring* ♕	1♕ 6♘♗		0	0.40	0.70	0.760
					Mean ± SE	0.46 ± 0.08	0.72 ± 0.01	0.723 ± 0.013

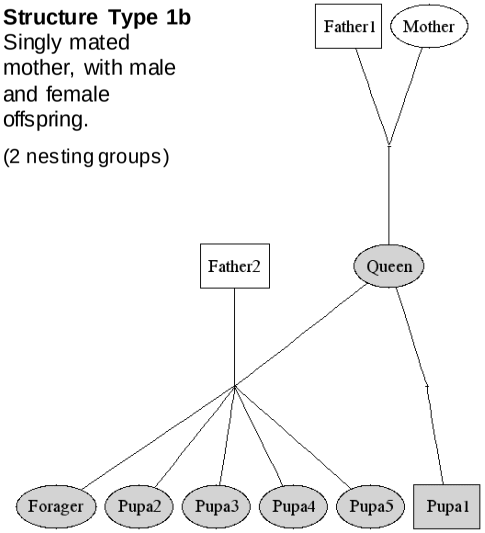
Structure Type 1a
Singly mated mother, with female offspring.

(3 nesting groups)



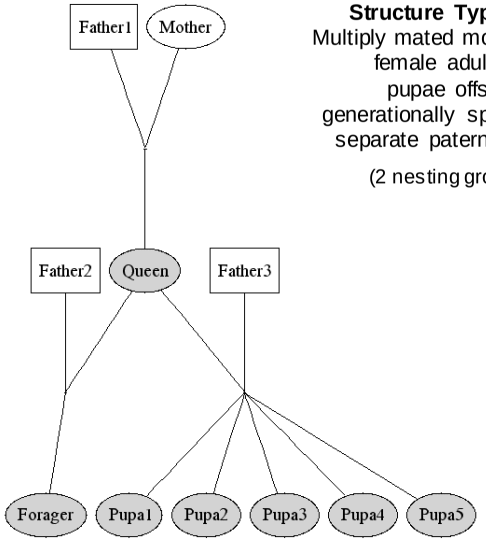
Structure Type 1b
Singly mated mother, with male and female offspring.

(2 nesting groups)



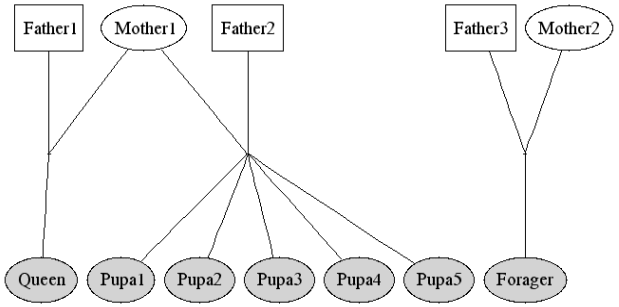
Structure Type 1c
Multiply mated mother, female adult and pupae offspring generationally split by separate paternities.

(2 nesting groups)



Structure Type 2a
Non-genotyped multiply mated mother, female adult and pupae offspring generationally split by separate paternities. Egglayer half-sister to pupae. Forager has different mother and father to nestmates.

(1 nesting group)



Structure Type 2a
Non-genotyped singly mated mother, with female offspring. Egglayer full-sister to pupae.

(1 nesting group)

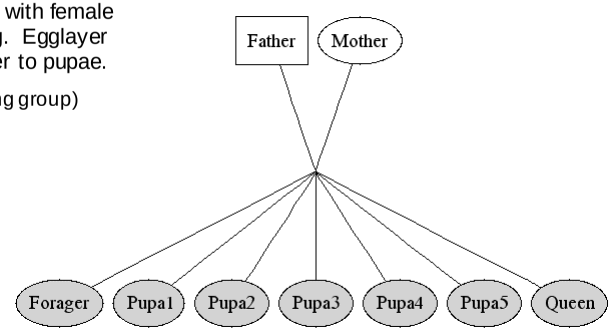


Figure 3.2. Family structures found in genotyped nesting groups (COLONY analysis). Legend: Queen/Forager - observed egglayer or forager by behavioural/census assessment; Pupa - sampled pupae; oval - females, rectangle - males; shaded shapes are actual genotyped samples. Structure Type 1, presumed resident queen present; Structure Type 2, possible queen turnover.

3.5. Discussion

Genetic markers provide fine-scale information on family structure, and contribute to the study of social evolution by providing a measure of reproductive skew and relatedness within groups. In the cosmopolitan paper wasp genus of *Polistes*, genetic structure data are lacking for tropical species. Here we analyse a genetic dataset of a tropical species, *P. lanio*, to achieve three aims. First (Aim 1), we identified 86 SNP markers which provided analysis of genetic structure within nests, with cross-over in the sister species *P. canadensis*. Second (Aim 2), through genetic analyses we found that reproductive skew and hence relatedness in post-emergence nests of *P. lanio* is high; this genetic-structure is similar to that of post-emergence nests of its sister species *P. canadensis*. Pupae belonged to a single mother in each nest, who held a reproductive monopoly despite nesting with multiple potentially reproductively potent adult females. High within-group relatedness suggests that individuals that do not directly reproduce may receive indirect fitness from raising related siblings. Finally (Aim 3), as in *P. canadensis* we detected a low level of multiple mating with up to two fathers in some matrilineal offspring. Detected multiple patrilines appear to be segregated by generation.

Our approach to identifying SNPs exploited existing transcriptome data for individuals of *P. lanio* and *P. canadensis* (Patalano et al., 2015; Moreno et al., in prep.). Since *P. lanio* and *P. canadensis* are sister species, we expected that some SNPs that were polymorphic in one species to be polymorphic in the other. Of the original set of 120 SNPs markers identified across the two species, 86 of them were polymorphic in *P. lanio*; 59 of these loci were polymorphic in the previously sampled *P. canadensis* population (Chapter 2, Appendix II).

High reproductive skew is typical in established post-emergence nests of temperate *Polistes* species, suggesting that conflicts over reproduction at this stage in the nesting cycle are resolved. As female workers are often offspring of the resident queen, and related to the brood, conflict over who reproduces may be reduced as workers gain increased indirect fitness from helping to raise siblings (Field et al., 1998; Reeve et al., 2000; Seppä et al., 2002). This current study on *P. lanio*, together with the parallel study on its sister species *P. canadensis* (Chapter 2), provides compelling evidence that tropical species of *Polistes,* despite differences in ecological conditions, also experience similar resolution of reproductive conflict as in temperate species of *Polistes*.

The observation a reproductive monopoly in nests of both *P. lanio* and *P. canadensis* (Chapter 2) raises questions about the mechanism/s of queen control over worker reproduction in *Polistes*, which is thought to be primarily via suppression through physical aggression (Jandt

et al., 2014). Control of reproduction by workers is not thought to have evolved in these simple eusocial societies. Queens in annual temperate species live in colonies that are small enough that the queen may maintain aggressive interactions with each worker as required (Trivers & Hare, 1976; Brian, 1980). The nesting groups in tropical species typically grow much bigger than those found in temperature counterparts (West-Eberhard, 1969; Pickering, 1980; Judd, 1998; O'Donnell & Joyce, 2001); for example, in our study on *P. canadensis* the three largest sampled post-emergence nests (by total number of cells) had a mean number of 648 cells and 48 adults present on the nest (Chapter 2). Large post-emergence nests of *P. canadensis* have also been observed across Panama, of up to 812 cells in size (Pickering, 1980). In comparison, though sizes will vary between geographical distributions and year, the widespread temperate *Polistes dominula* has been found with post-emergence nests of a mean 249 cells, whilst end of season temperate *Polistes fuscatus* nests were found with a mean 274 cells, and *Polistes instabilis* 283 cells (Downing & Jeanne, 1987; Baracchi et al., 2010). The three largest sampled post-emergence nests in our *P. lanio* were relatively small, with a mean number of 206 cells and 20 adults present, however larger nesting groups were observed in other Trinidad populations (the three largest *P. lanio* post-emergence nests by cell number, observed in Chapter 6, had a mean of 432 cells and 32 adults present, Table 6.1). It has been suggested that queens lose control over worker reproduction when colonies become too large, as they are physically unable to maintain aggressive interactions with all workers; this was demonstrated by queen tethering experiments which resulted in secondary egglayers (West-Eberhard, 1986). However, our analyses suggest that queens across established colonies do not lose control despite the number of adult females present, and are able to monopolise both female and male production. Future work should seek to determine to what degree (if any) tropical *Polistes* use pheromones to signal fertility, and whether they employ other mechanisms besides physical dominance to suppress worker reproduction (West-Eberhard, 1969; Kasuya, 1983; Izzo et al., 2010; Hunt & Richard, 2013; Oliveira et al., 2017).

We detected signs of queen turnover in two of the nesting groups, as the genotype of the egglayer did not match that of the brood mother. Queen turnover is common in simple eusocial societies of bees and wasps: if the original queen disappears or becomes weak then another female worker, usually of a high rank (in a linear hierarchy), may inherit the nest as a new queen. Queen turnover may be detected when the older and younger offspring belong to different matrilines, as seen in previous genetic studies on *Polistes* (Strassmann & Meyer, 1983; Hughes et al., 1987; Peters et al., 1995). Although loss of a queen can be costly (Strassmann et al., 2004), queen turnover allows the nesting group to continue and provides an incentive for distantly related individuals to remain on the nest and help, as there is a chance to inherit a reproductive monopoly if the resident queen dies (Field & Leadbeater, 2016). In other more complex eusocial species, such as bumblebees *Bombus spp.* and honeybees *Apis mellifera*, queen loss may signal the termination of the nesting cycle as

workers are limited to producing only male offspring (Duchateau & Velthuis, 1989; Pirk et al., 2004). An alternative explanation for our results, is there may have been multiple queens (West-Eberhard, 1986): as we genotyped only five brood per nest we lack the sample size to exclude this possibility. As all the adults on the nest were not genotyped, we may have also inadvertently sampled a subordinate 'sneak' egglayer in behavioural observations, whose eggs would likely be removed via queen policing before they developed to pupae (West-Eberhard, 1969).

Female multiple mating was detected in three matrilines, with a second patriline detected in each case. Overall effective mating frequencies were (mean ± se) 1.15 ± 0.08 in *P. lanio*; this is slightly lower than that found in *P. canadensis* 1.22 ± 0.07 (Chapter 2), but higher than in most Polistines being 1.01 (reviewed in Strassmann, 2001). However, the estimate of overall effective mating frequency in *P. lanio* is not significantly different from single mating; multiple mating may therefore be facultative. Patterns of patriline assignment in the sampled *P. lanio* population appeared to be segregated by generation, such that the brood belonged to one patriline and the adults to another. Genotyping errors are unlikely to explain this pattern, as our genotyping error rates were low (there was no mismatching with positive controls), and we used a conservative error chance in COLONY pedigree analysis of 1% across loci. However, we only sampled a small number of adults, and so to accurately explore this potential generational split by paternity more individuals should be analysed. In the Hymenoptera, although sperm from one partner may be preferentially used over the other, sperm is likely to be ultimately mixed in the spermatheca and used randomly in egg fertilization rather than clumped with temporal sequential fertilisation of eggs (the latter being rarely observed in some species) (Cole, 1983; Strassmann, 2001; Stürup et al., 2014). Sequential mating may explain the observed paternity patterns, whereby a female may mate with another male later in life, but this is expected to be rare in the Hymenoptera as reproductive males must either enter the nest past female workers (additional queen-mating would decrease sister relatedness) or a queen would have to leave the protection of the nest to mate (Strassmann, 2001). Multiple mating is not predicted to occur in the simple eusocial societies. This is because multiple mating lowers the relatedness between helpers and the brood they help raise. Multiple mating is therefore a derived trait that may be evolutionary stable once species have 'passed the point of no return' in social evolution, when workers are no longer able to fully reproduce (Hughes et al., 2008; Boomsma & Gawne, 2017). Our observation of multiple mating in both *P. lanio* and *P. canadensis* (Chapter 2) highlights the question of whether *Polistes* are indeed eusocial insects (in which females are expected to mate during a single event in their life) or cooperative breeders (in which females may remate each breeding season). It has been suggested that the simple eusocial societies of *Polistes* represent cooperative breeders, as they form semi-social associations during nest founding, and because workers are not committed to a life of investment in indirect fitness (Boomsma, 2009). The

discovery of multiple mating in *P. lanio* and *P. canadensis* (Chapter 2) is the first firm evidence that *Polistes* may be a cooperative breeder; sequential mating is not attainable in temperate *Polistes* as mating is time limited by seasonality (Lucas & Field, 2013). In the related tropical *Ropalidia marginata*, which has been described as a cooperative breeder much like *Polistes spp.* (Boomsma, 2009), there has also been observations of female multiple mating with up to three partners (Muralidharan et al., 1986; but evidence is not fully clear, see Shilpa et al., 2012). Further work is required to determine to what extent low levels of multiple mating are ubiquitous in tropical *Polistes*, and to what extent temporal segregation of patriline contributions are real.

In conclusion, we present evidence that the nesting group genetic structure of the tropical *P. lanio* is conducive to generating significant indirect fitness payoffs for non-reproductive nestmates, as well as potential direct fitness payoffs through inheritance as a replacement queen in turnover. Genetic structure data contributes to further knowledge on *Polistes* behavioural ecology, and the evolution of sociality in this cosmopolitan genus.

3.6. Appendix List

Associated appendixes: Appendix II – SNP loci information; Appendix IV – alternative COLONY analysis.

Chapter 4: Long live the wasp: adult longevity in captive colonies of the eusocial paper wasp *Polistes canadensis*

This chapter is formatted from the published paper: Southon, R.J., Bell, E.F., Graystock, P., & Sumner, S. (2015). Long live the wasp: adult longevity in captive colonies of the eusocial paper wasp *Polistes canadensis*. *PeerJ*, 3:e848.

Contributions: R. Southon, E. Bell, P. Graystock, & S. Sumner designed the study; R. Southon & E. Bell conducted the fieldwork, with help from field assistant D. Fabbro; R. Southon & E. Bell conducted experiments; P. Graystock analysed the results with help from R. Southon and E. Bell; paper was jointly written by R. Southon, E. Bell, P. Graystock, & S. Sumner.

4.1. Abstract

Insects have been used as an exemplary model in studying longevity, from extrinsic mortality pressures to intrinsic senescence. In the highly eusocial insects, great degrees of variation in lifespan exist between morphological castes in relation to extreme divisions of labour, but of particular interest are the primitively eusocial insects. These species represent the ancestral beginnings of eusociality, in which castes are flexible and based on behaviour rather than morphology. Here we present data on the longevity of the primitively eusocial Neotropical paper wasp *Polistes canadensis*, in a captive setting removed of environmental hazards. Captive *P. canadensis* had an average lifespan of 193 ± 10.5 days; although this average is shorter than most bee and ant queens, one individual lived for 506 days in the lab – longer than most recorded wasps and bees. Natal colony variation in longevity does exist between *P. canadensis* colonies, possibly due to nutritional and genetic factors. This study provides a foundation for future investigations on the effects of intrinsic and extrinsic factors on longevity in primitively eusocial insects, as well as the relationship with natal group and cohort size. As males are short-lived in the Hymenoptera, male helping effort (see Chapter 6) may be assured by long-lived females and by extension long-surviving colonies, after male departure or death.

4.2. Introduction

Death comes to all, yet many seemingly ordinary insects have evolved some of the most dramatic and extraordinary lifespans, delaying the call of death for remarkable periods (Finch, 1990). Variation in insect longevity spans from *Ephemera simulans* males that live as adults for just 1.6 days (Carey, 2002) to the ants *Pogonomyrmex owyheei* and *Lasius niger* whose queens can live up to 30 years (Porter & Jorgensen, 1988; Hölldobler & Wilson, 1990). Interestingly, eusocial insects such as ants, wasps, and bees feature heavily as examples of long-lived insects, but great variation exists not only between these species but also within species and even among genotypes. We understand little about the roles of ecology, evolution, life history, and environment in generating variation in longevity in social insects, largely due to the difficulty of disentangling intrinsic life-span (hence-forth referred to as longevity) from survival (the abiotic and biotic environment pressures i.e., extrinsic mortality) on individuals (Hölldobler & Wilson, 1990; Keeler, 1993; Keller, 1998; Giraldo & Traniello, 2014).

Eusocial insects are one of the most dominant, prolific, and diverse groups of organisms on the planet (Wilson, 1975). Much of this groups' success is attributed to the division of labour within the colony in the form of castes, with few or a single reproductive individual (*queen)*, supported by tens to millions of non-reproductive individuals (*workers)* that forage, provision, and care for sibling brood (Crespi & Yanega, 1995). Caste fate is primarily determined by environmental conditions, e.g., nutrition during larval development, and occasionally genetic biasing (Oster & Wilson, 1978; Hölldobler & Wilson, 1990; Hughes et al., 2003). Within species, variation in longevity can be pronounced between castes, with queens living as much as 100-fold longer than their related workers (e.g., general: Kramer & Schaible, 2013; Lucas & Keller, 2014; ants: Hölldobler & Wilson, 1990; Keller & Genoud, 1997; wasps: Ridley, 1993). This is a remarkable example of a how a single genome can display plasticity in aging (Keller, 1998; Keeler, 1993; Fjerdingstad & Crozier, 2006). Few individuals are selected to specialise in egg production and therefore colony survival is likely to be highly associated with and dependent on queen longevity (in the absence of reproductive succession, see Bourke, 2007). As a result, specialised egg layers are frequently protected from extrinsic pressures such as predation, for example the long-lived queens of the Harvester ant (*Pogonomyrmex owyheei*) live deep within the nest where they are sheltered (Porter & Jorgensen, 1981). Assuming there are costs associated with longevity (e.g., nutritional demands during larval development, development time), evolutionary theory would predict there would be selection for short lifespan in workers, and long-life span in queens, especially in highly eusocial species where colonies are large enough to support highly specialised, short-lived workers (Evans, 1958; Carey, 2001; De Loof, 2011; Ferguson-Gow et al., 2014). This has been shown to be the case with weaver ants in a protected lab environment whereby major workers (who take on more risky tasks) have a shorter intrinsic lifespan than minor workers who adopt less risky tasks (Chapuisat & Keller, 2002). The level of social complexity appears to be an important

predictor of longevity in the eusocial insects. Castes are unlikely to have been selected for such differential longevity in the primitively eusocial species, where colonies are small, each worker is valuable, and survival of workers may be highly variable depending on the type or frequency of task each individual performs (Strassmann, 1985).

Between species, individual longevity is often correlated with mature colony size, as shown in several wasp (*Vespa spp.*) and ant (*Myrmica*, *Leptothorax*, *Solenopsis*, *Cataglyphis*) species (Matsuura & Yamane, 1990; Schmid-Hempel, 1998). In ants, at the colony level, the first worker brood are often physically smaller with shorter lifespans than those produced later in the colony cycle, such as nanitic workers of ants (Porter & Tschinkel, 1986). It is hypothesised that this may be due to the increase in levels of nutrition available to brood as the colony grows (Oster & Wilson, 1978; Porter & Tschinkel, 1986). As the colony grows, the ratio of workers to larvae often increases, the larvae will then benefit from increased quality and quantity of food, which can result in longer adult life-spans (e.g., in honey bees *Apis mellifera*: de Groot, 1953; Eischen, 1982). Conversely in the primitively eusocial paper wasp *Polistes exclamans*, Strassmann (1985) identified that late emerging workers survived less time than early emerging workers in 1977 and 1978. This pattern was not detected in 1979 and its cause was suggested to be due to extrinsic factors.

Extrinsic factors such as parasitism, prey availability, and abiotic conditions can be powerful determinants of survival to wild individuals (Gibo & Metcalf, 1978; Strassman, 1979; Strassmann, 1981a; Tibbetts & Reeve, 2003). To date there have been no studies on how individual longevity varies with colony size in primitively eusocial insects in the absence of such extrinsic factors. Based on the larval nutrition quality to adult longevity theory, we predict the same patterns will occur as in the highly eusocial species, since individuals emerging early in the colony cycle are subject to low worker: larvae ratio and therefore low quality nutrition. Conversely, those emerging late in the colony cycle experience high worker: larvae ratio and thus high quality nutrition (Sumner et al., 2007). Additionally, there may be a genetic link to longevity whereby some colonies are more likely to produce long living individuals than others, potentially due to heritable differences in feeding/hunting propensity (VanRaden & Klaaskate, 1993; Herskind et al., 1996; Vollema & Groen, 1996; Klebanov et al., 2001; Sebastiani et al., 2012; Gems & Partridge, 2013). If colony effects are important, we predict that variation in longevity will be greater between colonies than within colony, even in the face of group size variation. Finally, positive correlations between colony size and longevity may be due to social-behavioural and metabolic factors such as increased per capita work rate in small colonies (Karsai & Wenzel, 1998).

Here we provide primary data on longevity of females in captive colonies of the predatory and primitively eusocial Neotropical *P. canadensis* paper wasp. Primitively eusocial species, such as those of the paper wasp genus *Polistes*, have been used to extensively study the evolution

of eusociality, with their lack of morphological differences and plasticity in caste (Turillazzi & West-Eberhard, 1996; although see Hunt, 2006). Although some studies have addressed the survivorship and colony phenology of some *Polistes* species (e.g., O'Donnell & Jeanne, 1992b; Giannotti, 1997; Clapperton & Dymock, 1997), there remain few systematic attempts to quantify longevity, and variation of, in this well-studied genus. Many tropical Polistinae such as *P. canadensis*, although influenced by wet/dry seasonality in food abundance (and resulting colony productivity), mate and have colonies of various life-stages throughout the year (Pickering, 1980; Clutton-Brock, 1991). These study systems offer an excellent system for testing the influence of ecology, evolution, and environment on longevity, in the absence of seasonal curtailment of longevity found in temperate species. Studying insect lifespans in captivity, in the absence of predation and parasitism, is a valuable approach that allows us to quantify longevity in the absence of extrinsic mortality pressures (Chapuisat & Keller, 2002). We assess how natal colony size correlates with longevity under laboratory conditions and follow this up by comparing longevity in experimentally manipulated group sizes. Understanding variation in longevity in these organisms provides an excellent foundation to explore similar questions in the higher-order social vertebrates (Carey, 2001).

4.3. Methods

4.3.1. Collection

We collected ten colonies (M1–M10) of the paper wasp, *P. canadensis* from the Province of Colón in Republic of Panamá in August 2013 for transportation to the UK (9°24′03″N 79°52′11″W). Adult wasps were captured with full nest carton containing brood (eggs, larvae, and pupae) during dusk. The nest cartons and wasps were transferred to individual containers (15 cm x 15 cm x 15 cm) with wire mesh ventilation. Colonies were then provided with sugar solution and water *ad libitum* during transfer to the United Kingdom in luggage kept at ambient temperature. Turnaround from capture to settled maintenance in the laboratory was 48 h. To ensure that no colony was heavily infected with entomopathogens from the field, a subset (5 individuals per colony) of cadavers were placed in isolated petri dishes to observe any resulting sporulation of any infecting fungal entomopathogens. The common fungal agent *Aspergillus spp.* sporulated from 10% of these cadavers. *Aspergillus* is an opportunistic and largely ubiquitous fungus, commonly regarded as non-lethal to social insects unless under extreme stress or ingested at unnaturally high concentrations (Bailey, 1968; Foley et al., 2014).

4.3.2. Maintenance

Once in the UK, we housed nests in clear transparent acrylic containers 30 cm × 33 cm × 34 cm each with two 525 mm perimeter ventilation ducts (Figure 4.1). The food provided consisted of liquid cane sugar and live wax moth larvae *Achroia grisella*, along with distilled water and nest-building materials (cardboard & paper) all were supplied *ad libitum*. All sugar and food was obtained in batches and haphazardly split between colonies to ensure equal food quality provided to the adults to prevent any longevity variability as a result of adult nutrition (Johanowicz & Mitchell, 2000; Harvey et al., 2012). In addition to food, in each nest-box we provided plastic artificial planting in the form of a strip of 7 cm × 7 cm × 2.5 cm grass *sp.* and a 5 cm × 5cm × 5 cm plastic *Hedera sp.* for environmental enhancement to provide shelter from female aggression for males (Polak, 1993). The nest boxes were cleaned regularly with distilled water without disturbing wasps or nest. Natural conditions from the collection sites were mimicked with temperatures of 25 ± 1 °C, 70 ± 5% relative humidity, and a light cycle of 12 h light (12 h dark).

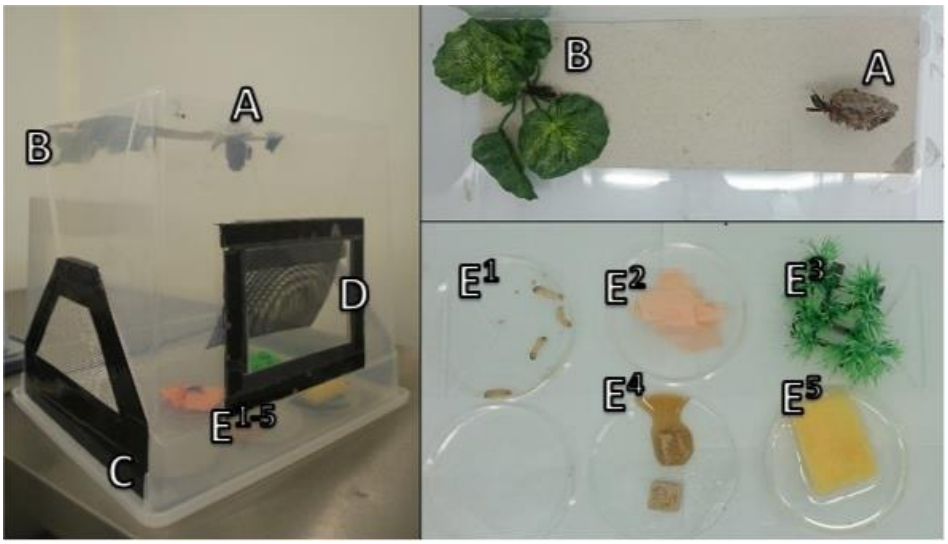


Figure 4.1. Captive housing of *P. canadensis* colonies. A, Nest carton supported on reinforced celling with shade; B, artificial planting; C, ventilation; D, access hatch; E^1, food provisions; E^2, construction paper; E^3, artificial planting; E^4, liquid sugar cane; E^5, distilled water (Southon et al., 2015).

4.3.3. Data collection

The colonies were surveyed three to four times a week and any deceased adults found immediately removed from the nest box. Total deaths per colony/cohort were tallied on a weekly basis and this recording method continued until all individuals were deceased. Whilst newly laid eggs were left in the nest for the adults to tend to, brood were eventually removed before pupation ensuring that only the original adult wasps captured from the wild were monitored for longevity and the colony/cohort sizes remained constant. This ensured all of the adult wasps developed under semi-natural conditions. Since all nests were collected from the same field site at the same time, local environmental conditions for development are controlled for as best as possible, though the colonies will differ from each other genetically.

As we do not know the eclosion date for each adult wasp, measures of longevity will be underestimates.

4.3.4. Test 1: Adult longevity of female *P. canadensis* between colonies

Using the data generated from colonies M1–M7, we were able to quantify variance in longevity between colonies to determine whether colony identity explains variation in wasp longevity better than colony size. Using average survival per colony, correlations between initial colony size upon permanent laboratory setup were investigated.

4.3.5. Test 2: Adult longevity of female *P. canadensis* within colonies

Three colonies (M8, M9, M10) were monitored for a period of 3 months, at which point their group sizes were of 28, 23 and 23 workers respectively. Each colony was then split, and randomly allocated between two new nest boxes lacking nest cartons, giving six new groups in total and consisting of 18, 13, 12, 8, 8 and 9 females. A non-natal male was also added to each of the new colony nest boxes so that females had the opportunity to mate. All cohorts then started to build nest cartons and lay eggs suggesting mating may have occurred. The colonies were maintained as above with wasp deaths monitored weekly for 220 days, at which point all individuals were deceased.

4.3.6. Statistical analyses

Differences in adult survival were analysed using Cox proportional-hazards regression models, with colony identity as a factor. Where differences in survival were found, we conducted pairwise comparisons between colonies using additional Cox comparisons. Pearson product-moment correlations were carried out to look for patterns between colony size and average colony longevity. Analyses were carried out in R 3.3.3 (R Core Team, 2017), using the 'survival' package (Therneau & Grambsch, 2000).

4.4. Results

4.4.1. Test 1: Adult longevity of female *P. canadensis* between colonies

Here we found that 57% of adult *P. canadensis* colonies maintained in the lab can survive beyond 365 days, providing data on longevity for 143 wasps in total (Figure 4.2). On average wasps lived for 193 ± 10.5 days, with the oldest individual living for 506 days (Appendix V). Colony identity has a significant influence on adult wasp longevity (Cox proportional-hazard analyses: Wald = 19.67, d.f. = 6, $p = 0.003$). There was no correlation between colony size and the colonies' average longevity ($r = 0.06$, $n = 7$, $p = 0.890$; Figure 4.3). Regular observations did not identify any behavioural differences between colonies. All colonies built nests, maintained social cohesion, and regular egg laying was observed throughout.

4.4.2. Test 2: Adult longevity of female *P. canadensis* within colonies

Group sizes ranged from 9 (M5) to 34 (M7) wasps with average longevity within different nests ranging from 130 ± 39.4 days (in M5) up to 206 ± 41.3 days (in M6). Comparing the mean adult longevity of colonies M1–M7 against their original size gives no clear association (Figure 4.3). Colonies M8–M10 showed no difference in survival prior to splitting (Wald = 1.29, d.f. = 2, $P = 0.525$; Figure 4.4). However, after splitting into 6 cohorts of variable size, females exhibited significantly different longevities (Wald = 14.21, d.f. = 5, $p = 0.014$; Figure 4.5). Using pairwise comparisons, cohorts from M9 (M9A & M9B) and from M10 (M10A & M10B) show no significant difference in patterns of adult longevity within natal colony identity (Wald = 0.05, $p = 0.824$; Wald = 0.33, $p = 0.568$; respectively), cohorts from colony M8 (M8A & M8B) do differ from each other and are, incidentally, the 2 cohorts with the largest size difference (Wald = 4.84, $p = 0.028$). Group size shows a positive correlation with longevity ($r = 0.84$ $n = 6$, $p = 0.038$; Figure 4.3). Regular observations did not identify any behavioural differences between cohorts. All cohorts built nests, maintained social cohesion, and regular egg laying was observed throughout.

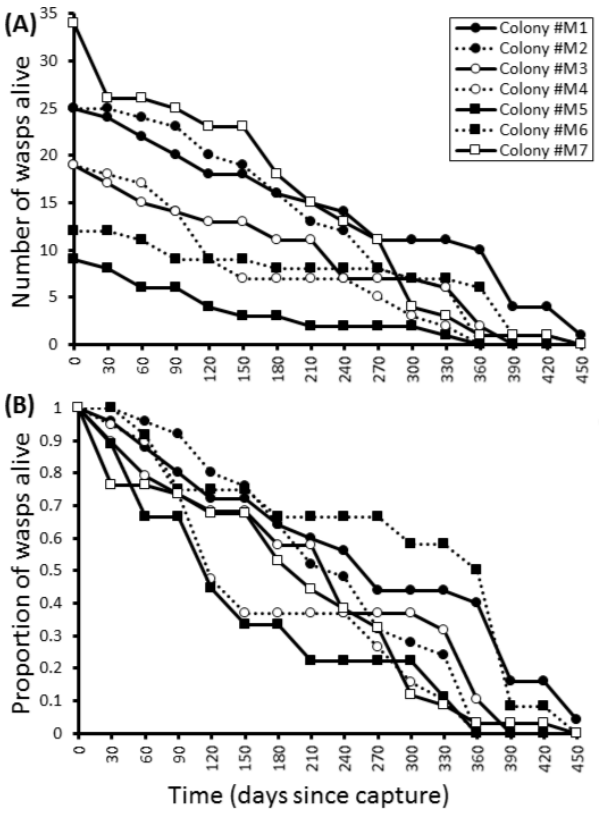


Figure 4.2. Survival details of seven colonies of *P. canadensis* over 450 days. Survival of adults in seven *P. canadensis* colonies shown as raw numbers (A) and proportions (B) over a period of 15 months post capture whilst maintained under laboratory conditions.

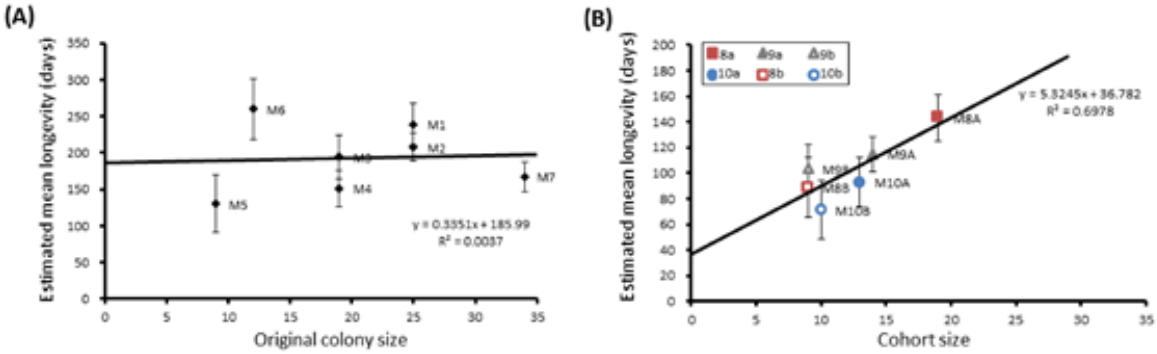


Figure 4.3. Mean longevity per colony against colony size. Correlations between colony size and mean longevity of adult *P. canadensis* when maintained in original colony (A) or when manipulated into cohorts of varying size (B). Standard error bars shown (Southon et al., 2015).

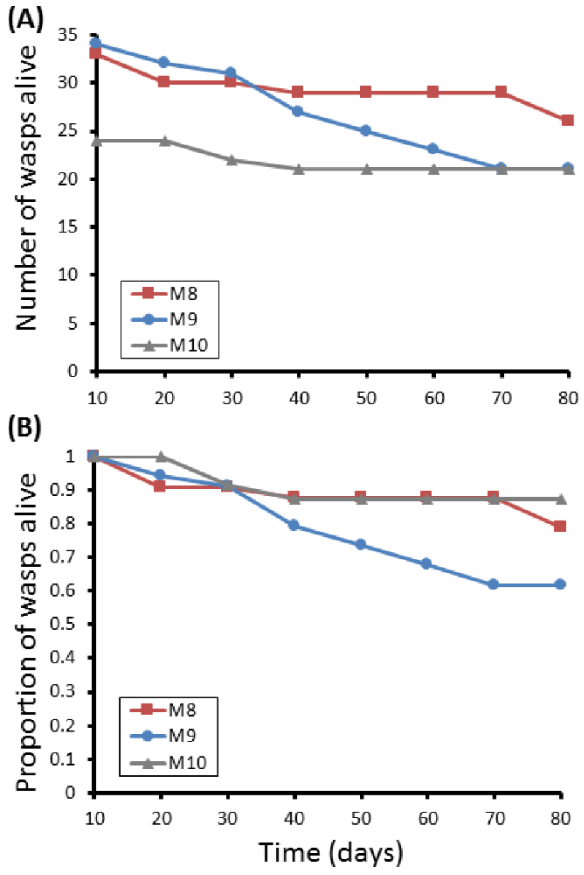


Figure 4.4. Survival details of three colonies of *P. canadensis* over 80 days. Survival of adults in three *P. canadensis* colonies (92 adults) shown as raw numbers (a) and proportions (B) over a period of 80 days post capture whilst maintained under laboratory conditions (Southon et al., 2015).

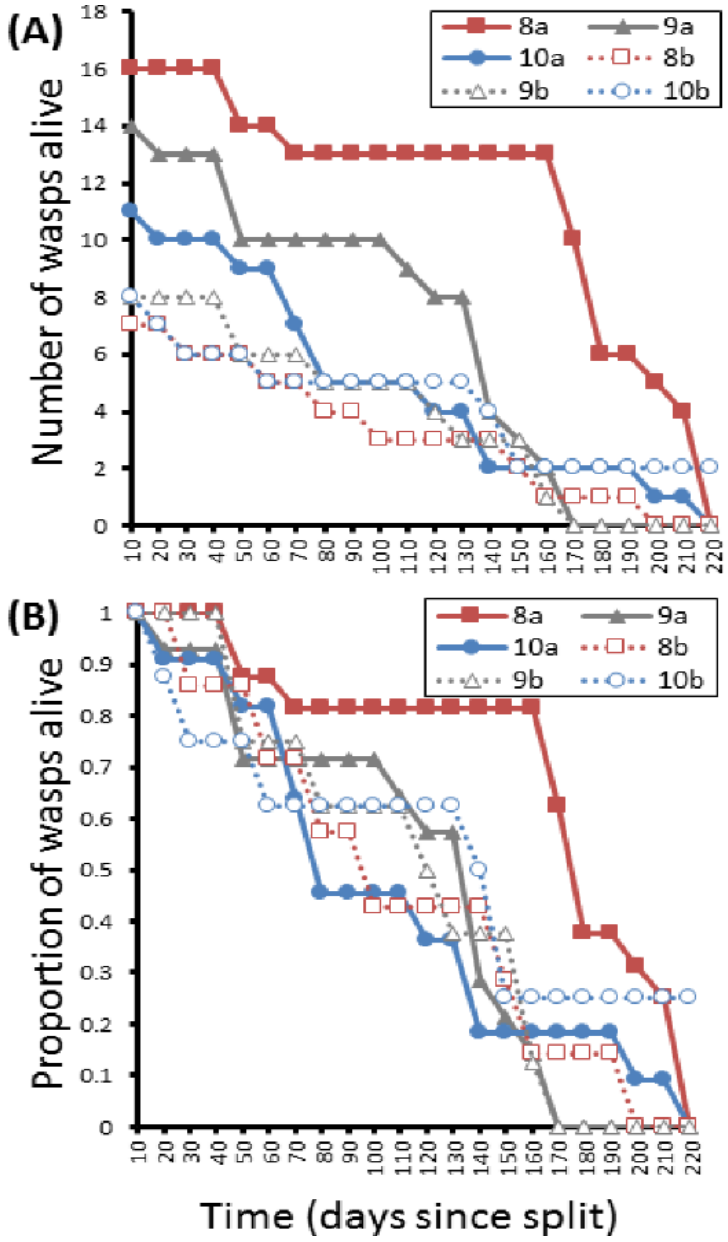


Figure 4.5. Survival details of six cohorts of *P. canadensis* wasps from 3 natal colonies. Survival of 72 adult *P. canadensis* in 6 conspecifics groups split from 3 colonies, shown as raw numbers (A) and proportions (B) over a period of 220 days post split whilst maintained under laboratory conditions. Significant difference ($p < 0.05$) in Cox proportional-hazards between colony split M8a and M8b (Southon et al., 2015).

4.5. Discussion

Here we show that with an average lifespan of 193 days, *P. canadensis* have some of the longest lifespans of recorded wasps under laboratory conditions to date. The oldest wasp in our study lived for a staggering 506 days which is one of the longest living lab assisted, or wild recorded wasp – and most recorded wild and assisted bees, with the notable exception of assisted honeybee queens tending to live around live around 3,000 days (Figure 4.6). The natal group was a significant predictor of longevity with wasps living on average between 260 days and 130 days depending on nest, though the size of the natal groups had no correlation with longevity. When natal groups were split in to varying cohort sizes, the largest cohort lived the longest and a correlation between longevity and cohort size was identified. We discuss the implications of these data in the context of other species and ecology and evolution of eusociality.

We observed large cohorts of long lived individuals in multiple colonies, although we cannot say whether individuals were of queen, worker, or in a quiescence (Hunt, 2006) status/state. In the most general sense, eusocial structure is based on behaviour rather than the physiological constraints observed in more highly eusocial hymenopterans such as honey bees and many ants, which incidentally display the largest longevity disparities between castes. Though there are no directly comparable studies that use *Polistes* in a lab setting such as this study, the lifespan estimates on workers of wild or assisted *Polistes* species tends to be approximately one month, with the longest average life span being 37 days in *Polistes lanio* and shortest life span being 14 days in *P. exclamans* (Miyano, 1980; Strassmann, 1985; Giannotti & Machado, 1994b; Giannotti, 1997; Giannotti, 2012; Gamboa, Greig & Thom, 2002; de Oliveira Torres et al., 2013).

The average lifespan of egg laying *Polistes* wasps can range from a maximum of 209 days in wild *P. lanio* queens down to 66 days in wild queens of *Polistes fuscatus* wasps (Giannotti & Machado, 1994b; Gamboa, Greig & Thom, 2002). Whilst there is an observation from Phil Rau (West-Eberhard, 1969) of a marked wild temperate *Polistes* queen living for approximately 2 years, it seems tropical species may have some of the longest lifespans, although further empirical studies are needed to tease apart seasonal affects. In each case, the studies were carried out on wild *Polistes* colonies and so cannot account for extrinsic mortality (Strassmann, 1985; Giannotti & Machado, 1994b). The life-span of wasps in more highly eusocial species ranges from 1,000 days in queens of some wild *Vespa spp.*to 14.5 days in lab maintained *Vespula germanica* and *Vespula consobrina* (Akre, 1982; Dazhi & Yunzhen, 1989; Hölldobler & Wilson, 1990).

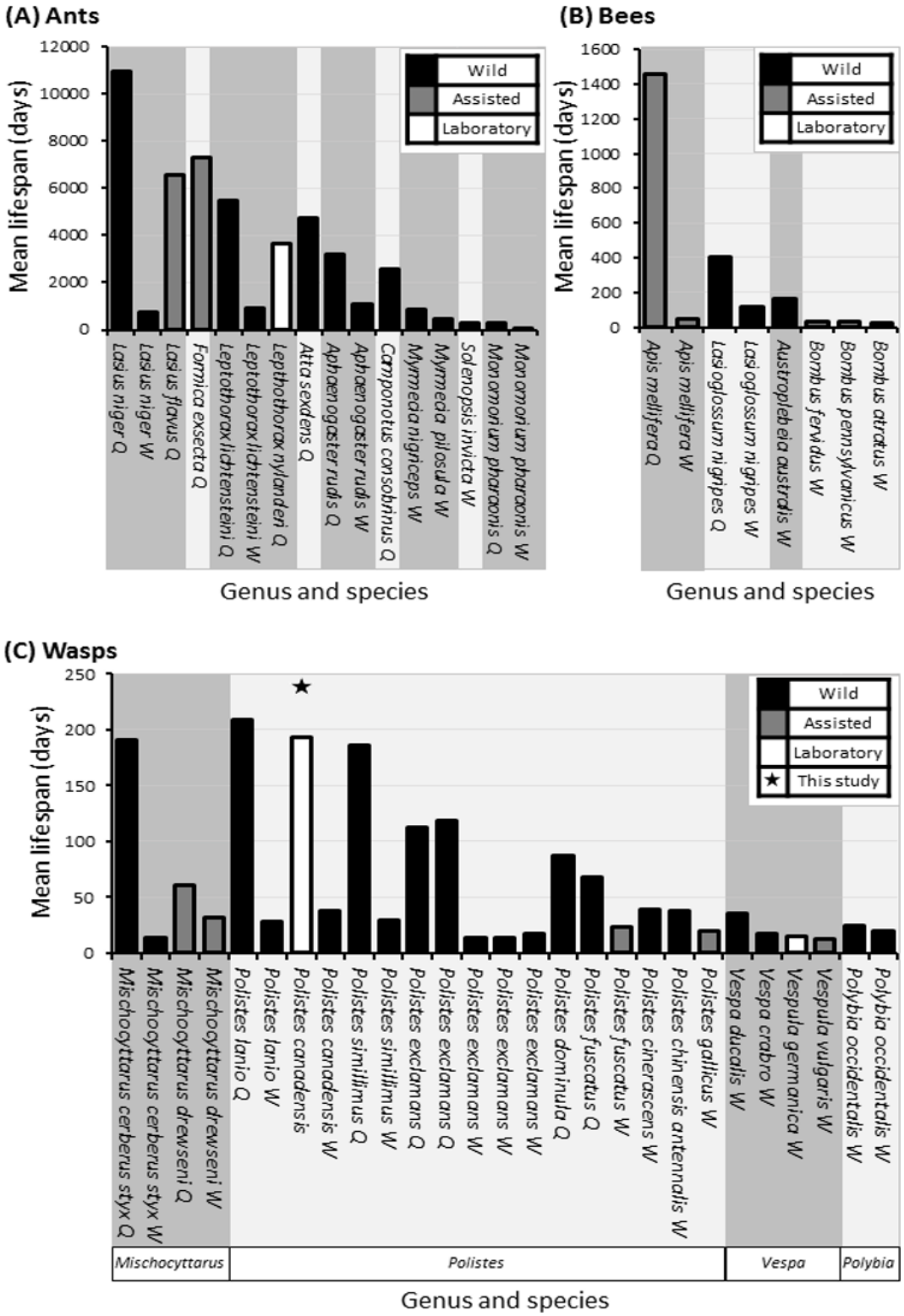


Figure 4.6. Comparative mean lifespans in ants, bees and wasps. Comparison of lifespans in a selections of ants (A), bees (B) and all records of wasps (C), highlighting whether data is attained from wild (Black bars) or assisted (grey bars) colonies with unknowns also included (grey bars). Queen lifespans in minimum age, as most studies/literature start from colony creation. Data compiled from the result of this study

(above column) and those found following literature search (Pardi, 1948; Michener, 1969; West-Eberhard, 1969; Matsuura, 1971; Wilson, 1971; Spradbery, 1973; Miyano, 1980; Haskins & Haskins, 1980; Akre, 1982; Strassmann, 1985; Goldblatt & Fell, 1987; Dazhi & Yunzhen, 1989; Hölldobler & Wilson, 1990; Pamilo, 1991; O'Donnell & Jeanne, 1992a; Giannotti & Machado, 1994a; Giannotti & Machado, 1994b; Keller, 1998; da Silva-Matos & Garófalo, 2000; Page Jr & Peng, 2001; Gamboa, Greig & Thom, 2002; Jemielity et al., 2005; Hurd, Jeanne & Nordheim, 2007; Archer, 2012; Giannotti, 2012; de Oliveira Torres et al., 2013; Halcroft, Haigh & Spooner-Hart, 2013; Jeanne, 1975) (Southon et al., 2015).

On average there is a positive correlation between the maximum lifespan of eusocial queens and the degree of eusociality displayed by that species (Carey, 2001; Kramer & Schaible, 2013) and differences found among species tend to be due to extrinsic mortality (Keller, 1998). Here we cannot differentiate between egg layers and helpers in our primitively eusocial species, yet evolutionary theory dictates that when a colony is small and the lifespans of both the reproductive individuals and helpers are equal, the helpers will resist evolutionary specialisation to workers as that would ultimately reduce their direct fitness potential (Alexander et al., 1991). We may therefore expect to find similar lifespans in both egg layers and workers in *P. canadensis*. However, differences in lifespan are observed between helpers and egg layers in other *Polistes* species (Figure 4.6). These studies use wild species though and describe the extrinsic mortality, unlike our study which had minimal extrinsic pressures.

Colony identity was a clear predictor of wasp longevity. All colonies were collected at the same time from the same field site, in which adults on all nests would have shared the same developmental and environmental conditions. The potential causes for the effect of colony identity could be: (1) genetic differences between the colonies. Genetic influences on longevity have been found in a number of model species from mammals to nematodes and insects (VanRaden & Klaaskate, 1993; Herskind et al., 1996; Vollema & Groen, 1996; Klebanov et al., 2001; Sebastiani et al., 2012; Gems & Partridge, 2013) and evidence for heritability of increased longevity in the fruit fly and honey bee has been observed (Rinderer et al., 1983; Luckinbill & Clare, 1985) with some gene expression patterns being associated with longevity in queen honey bees (Corona et al., 2005). (2) Queen 'quality' which can be the result of extrinsic or intrinsic factors. Variation in fecundity of reproductive and dominance over other individuals in a colony is known as queen quality and this can vary between queens (Harris & Beggs, 1995; Liebig et al., 2005; Holman, 2012). This queen quality variation can be inherited (Rinderer & Sylvester, 1978; Corona et al., 2005) or driven by environmental factors (Hatch et al., 1999; Tarpy et al., 2011). (3) Unobserved differences in extrinsic factors that the nests had experienced before collection. Since the colonies were not monitored for their entire life cycle, there is the possibility that something affected each one differently in order to cause

varying longevity within their workers. What we can conclude is that although colony genotype was a predictor of longevity in the adult wasps, this did not correlate with wasp group size unless the size was manipulated. This suggests that the colony influences are greater than those of group size, and whilst an overall correlation between manipulated group size and longevity was identified, only one out of three split colonies displayed this trend. This suggests that the explanation that larger colonies produce longer lived workers due to enhanced nutrition during larval development is not a major component.

To investigate the underlying variation in longevity in eusocial insects, data from captive colonies of a range of eusocial insects is a useful tool and can help uncover variation in investment for longevity based on extrinsic factors faced by a given species, individual, or caste (Chapuisat & Keller, 2002). Longevity studies on predatory eusocial insects such as wasps are underrepresented in the literature, and while several excellent studies have been identified, no studies have followed maintained *Polistes* in a protected lab environment. Here, for the first time we quantify longevity of adult *P. canadensis* in the absence of extrinsic mortality and provide some support for the link between group size and adult longevity but show that natal origin (i.e., genotype) is a more powerful predictor. Our results suggest that predictions founded on previous research using higher eusocial species such as honey bees may not be relevant to primitively eusocial species since their caste/fate is not fixed during development. A particular challenge will be for future studies to also control for all of the described extrinsic and intrinsic factors such as wild nest site condition and the presence of symbionts.

4.6. Appendix List

Associated appendixes: Appendix V – an old wasp.

Chapter 5. Delayed dispersal from the natal nest in *Polistes lanio* males is linked to sexual maturation and hormonal regulation of behaviour

Contributions: R. Southon, A. Radford, & S. Sumner designed the study; R. Southon conducted the fieldwork, with help from S. Sumner, summer intern J. Valverde, and field assistants L. Crowley & S. Morris; R. Southon conducted dissections, all other lab-work, and analyses.

5.1. Abstract

The social Hymenoptera typically exhibit sex-biased dispersal, as most newly emerged adult females stay with natal groups to help raise siblings, whilst males disperse to find mates. Within the framework of inclusive fitness theory, females in simple eusocial societies may choose between being either a philopatric helper (investing in indirect fitness), a replacement reproductive, or dispersing reproductive (investing in direct fitness). Males do not have this choice; they typically specialise in behaviours that promote direct fitness only – and this requires dispersal. However, in most social Hymenoptera, males delay dispersal from the natal nest after adult emergence. What processes regulate eventual male departure, and whether the proximate pathways that regulate this are conserved across the Hymenoptera, is largely unknown. Here we show that adult males on natal nests of the simple eusocial Neotropical paper wasp *Polistes lanio* are reproductively undeveloped, but there was a trend of increasing reproductive maturity with age in males that were still present on the natal nest. We also found that dispersed males were reproductively more mature relative to adult males which had not yet dispersed. Males treated with the juvenile hormone (JH) analogue methoprene departed from natal nests sooner than control groups, and possessed significantly more accessory gland fluid than control groups (a sign of reproductive maturity) after 48 hours. These results suggest that dispersal of developing adult males is regulated by a JH pathway. The ancestral function of JH in regulating male dispersal may be conserved across the Hymenoptera, as JH has been previously demonstrated to regulate male dispersal in Apidae bees. This study highlights the role of JH as a master regulator of insect behaviour in the male, as well as female, sex.

5.2. Introduction

Dispersal, the process whereby an individual permanently relocates, allows for gene flow across populations (Ronce, 2007). Dispersal may be beneficial in reducing the risk of inbreeding, and facilitates acquisition of alternative breeding or resource opportunities (Keller & Waller, 2002; Matthysen, 2012; Hansson & Åkesson, 2014). However, the benefits of dispersing may be negated by energetic and stochastic costs if performed under suboptimal conditions (Bonte et al., 2012). Patterns of dispersal vary considerably within and among species, as a response to internal state and/or the external environment (Bowler & Benton, 2005; Clobert et al., 2009). Determining patterns and processes of dispersal can help us understand the ultimate reasons why such behaviour has evolved.

Sex-biased dispersal is common across the animal kingdom, and may evolve from differing selective pressures between the sexes and from inbreeding avoidance (Trivers, 1972; Pusey, 1987; Prugnolle & de Meeus, 2002; Lawson Handley & Perrin, 2007; Gros et al., 2008). Species that show mate-guarding behaviour generally tend to have male-biased dispersal as males compete to gain access to females (e.g. in polygamous mammals), whilst species that show resource defence generally have female-biased dispersal as males are selected to attract mates to their patch (e.g. in monogamous birds) (Greenwood, 1980). Sex-biased dispersal patterns may influence social evolution, as philopatry promotes social interactions among members of the non-dispersing sex (Greenwood, 1980; Lehmann & Boesch, 2008; Johnstone et al., 2012; Nagy & Knörnschild, 2016). In the social Hymenoptera (ants, some bees and wasps), dispersal from the natal nest is typically male-biased (Hamilton, 1972; Jaffé et al., 2009; Johnstone et al., 2012). Daughters usually remain with natal groups as philopatric workers, gaining indirect fitness by helping raise relatives. Under certain circumstances, females may either become new replacement reproductives on the nest or disperse to found new nests, and thus gain direct fitness (Hamilton, 1972; Peeters & Ito, 2001; Deshpande et al., 2006; Jaffé et al., 2009; Johnstone et al., 2012; Vitikainen et al., 2015). By contrast, hymenopteran males usually always depart from natal nests soon after emergence as adults to search for mates (here we refer to adults as being the final holometabolic stage, whether functionally mature or not), and are primarily limited to gaining direct fitness within their short lifespan (Hamilton, 1964; Hamilton, 1972; Heinze, 2016). Thus, sex-specific dispersal in the social Hymenoptera is likely linked to contrasting (and male-limited) strategies to maximise fitness. Here we determine the timing of male-biased dispersal in a simple eusocial insect, and explore the proximate processes that may regulate it.

There is a trade off in the optimal timing of dispersal; too late and reproductive opportunities may be lost, whilst too early and wasted energetic costs may be excessive (Bonte et al., 2012). There will be selection for the dispersing sex to be able to use cues that maximise fitness payoffs of dispersal, such as in assessing reproductive maturity (of itself), or mate availability

in the surrounding environment (Clobert et al., 2009). Equally, group members delaying dispersal will be tolerated by non-dispersing relatives if it results in greater productivity of breeding relatives (Koenig et al., 1992). For example, delayed dispersal may be selected for when there is temporal variance in the trade-off between philopatric kin benefits and dispersal costs (Peer & Taborsky, 2007), or when dispersers can appease permanent residents by helping in the group until new breeding opportunities or sites are available for relocation (Komdeur et al., 1991; Koenig et al., 1992). In the social Hymenoptera, newly emerged males appear to delay dispersal until they reach sexual maturity (Hamilton, 1972; Moors et al., 2009). Delayed dispersal in adult males linked to developing maturation is likely associated with group living in the Hymenoptera; in contrast, it appears that males of solitary Hymenoptera (at least in the parasitoids) vary in whether they emerge as fully mature adults or not (Hagen, 1953). By remaining on the natal nest until reproductive maturity, temporary-social males may benefit from the added protection and nutritional resources gathered from other group members (such as those provided by worker-caste females), and receive accelerated rates of maturation, better final mating condition, and reduced risks of mortality (e.g. from predation) (Litte, 1977; Hunt et al., 1982; O'Donnell & Jeanne, 1992a; Leatemia et al., 1995; Yuval et al., 2002; Costamagna & Landis, 2004; Hunt, 2007).

Given the strong correlation between sexual maturation and dispersal in males, it is likely that the same hormones which regulate maturation also regulate dispersal. Hormones act as coordinators between physiological functions such as metabolism, growth, reproduction, and subsequent behavioural actions (Bolander, 2004). In mammals, hormones may regulate sex-biased or phenotype-specific dispersal such that one sex disperses more than the other, e.g. dispersing males in Belding's ground squirrel *Urocitellus beldingi* (Nunes et al., 1999); or both a philopatric and dispersal phenotype is present within a sex, e.g. morphologically and behaviourally distinct reproductive dispersers in naked mole-rats *Heterocephalus glaber* (O'Riain et al., 1996). In some insect species, hormonal changes during development may result in elaborate alternative dispersal phenotypes. For instance, in crickets *Gryllus spp.*, hormones mediate the development of long-winged dispersing and short-winged non-dispersing morphs (Zera & Zhao, 2003). Hormonal changes may also influence post-metamorphose adult behaviour in insects, promoting or ceasing dispersal through muscle re/de-generation and reproductive fecundity, depending on changing environmental conditions (Dingle & Winchell, 1997). Insect dispersal and reproductive maturation may involve a variety of hormones, such as the adipokinetic hormone and parsin neurohormone, but they are primarily thought to be regulated by the juvenile hormones (JH) (Rankin & Burchsted, 1992; Hartfelder, 2000; Ramenofsky & Wingfield, 2007).

JHs are a group of sesquiterpenoids that are likely to be master regulators of dispersal behaviour across the insects. JHs play an important role in a wide range of developmental and behavioural processes in insects, including reproduction, diapause and polymorphisms.

During pre-adult development, JH is involved in regulating metamorphosis (Riddiford & Truman, 1993). After adult emergence, JH performs a secondary function in regulating sexual development and behaviour in females and males (Wyatt & Davey, 1996; Goodman & Cusson, 2012). JH, specifically JH3, is strongly associated with dispersal flight behaviour in female Hymenoptera (Hartfelder, 2000). However, whether JH promotes or ceases dispersal varies across insect taxa, and is likely dependent on differing selection pressures (Rankin & Riddiford, 1978; Giray & Robinson, 1996; Dingle & Winchell, 1997; Hartfelder, 2000; Zera & Cisper 2001; Sasaki & Nagao, 2013).

In females of the social Hymenoptera, the ancestral function of JH is as a regulator of reproductive physiology. For example, in the female sex of many Hymenoptera, JH upregulation (or experimental application of JH analogues) promotes dominance behaviour and ovarian development in the individual (*Polistes spp.* – Barth et al., 1975; Röseler et al., 1984; Giray et al., 2005; Tibbetts & Izzo, 2009; *Bombus terrestris* – Bloch et al., 2000; *Megalopta genalis* – Smith et al., 2013). However, in some complex eusocial species such the honeybee *Apis mellifera*, JH does not appear to regulate reproductive physiology in either adult queens or female workers and has hence lost its ancestral function (Robinson et al., 1991; however, JH still behaviourally regulates gyne mating flights – Hartfelder, 2000). The secondarily derived function of JH in the social Hymenoptera is in the regulation of female worker behaviour, correlated with age polyethism in which specific labour tasks such as nursing and foraging are distinctly split between age groups. Applications of the JH analogue methoprene is shown to accelerate the transition rates of worker age polyethism in the simple eusocial species of *Polistes canadensis* and *Polistes dominula* (Giray et al., 2005; Shorter & Tibbetts, 2009), and in the complex eusocial species of *Polybia occidentalis* and *A. mellifera* (Robinson, 1987; O'Donnell & Jeanne, 1993). This influence of JH is varied, as JH appears not to be associated with worker age polyethism in the simple eusocial paper wasp *Ropalidia marginata* (Agrahari & Gadagkar, 2003).

Given the functional diversity of JH in social hymenopteran females, an outstanding question is whether JH is a key regulator of behaviour and reproduction in male counterparts. To our knowledge, the role of JH in regulating reproductive physiology and behaviour of hymenopteran males has only been investigated in the Apidae bees, restricted to two species – the solitary carpenter bee *Xylocopa appendiculata*, and social *A. mellifera*. In Apidae males, JH appears to have retained its ancestral function in regulating gonadotropic maturation processes and subsequent dispersal behaviour (Giray & Robinson, 1996; de Oliveira Tozetto et al., 1997; Harano et al., 2008; Sasaki et al., 2012; Sasaki & Nagao, 2013). Whether JH has retained similar ancestral functions in males of other (non-Apidae) families where separate linages of sociality have evolved (Hughes et al., 2008), such as the social Vespidae wasps, is unknown.

Here we examine the timings and processes of male dispersal in the simple eusocial Neotropical paper wasp *Polistes lanio*. Males of *P. lanio* make an ideal model for determining dispersal processes as both males and females emerge throughout the year in the tropics, and so dispersal by males is not strictly time or mate-limited (Giannotti & Machado, 1994a; Lucas & Field, 2013). Using natural dispersal observations and experimental applications of the JH analogue methoprene, we aimed to determine the timing of adult male dispersal from natal nests and the potential role of JH. Specifically, we addressed three main questions. First, is male dispersal from the natal nest age-determined, irrespective of variation in traits of natal nests? Second, do males delay dispersal until they are sexual maturate? Finally, does JH regulate male natal dispersal (physiologically and behaviourally)?

5.3. Methods

5.3.1. Study sites

Post-emergence nests (established nests with emerged adult offspring) of *P. lanio* were studied during the tropical wet season in Trinidad, Trinidad & Tobago, June–August 2014 and July–September 2015. Nests were located in semi-rural areas, along the eaves of houses, in abandoned buildings, livestock pens, and bushes. A total of 37 nests were used from four sites that were utilised throughout the thesis (of approx. two hectares each): 22 nests at Verdant Vale (site VV), Blanchisseuse Rd, 2014–15 (10°41'5.44"N, 61°17'24.95"W); nine nests at Eastern Main Rd (site EM), 2014–15 (10°39'1.21"N, 61°15'9.63"W); four nests at Cumuto Tamana Rd (site CT), 2015 (10°34'48.01"N, 61°14'38.06"W); and two nests at the University Field Station of the University of the West Indies (site UWI), San Juan-Laventille, 2015 (10°38'16.04"N, 61°25'37.94"W).

5.3.2. Question 1: Is male dispersal from the natal nest age-determined?

To investigate the timing of natal male dispersal, we monitored natural dispersal rates of males by age across nests.

Sample collection: The ages of 154 natal males at dispersal across 27 nests at sites VV, EM, and CT were recorded between June and September in 2014 and 2015 (Table 5.1). Each nest was monitored daily throughout this observation period. At the start of the monitoring period, all male wasps were individually marked. For marking, each wasp was taken off the nest with forceps, and a four-coloured spot combination applied onto the dorsal thorax using extra fine tip Uni POSCA markers. Wing length (as a straight line between the intersection of a tegula and wing to the furthest apex tip) was measured with digital calipers in millimetres (to 2 d.p.). If wings were creased, as is typical in recently emerged adult wasps, wings were unfolded before measurement. Wing length is a reliable indicator of overall body size (in females, West-Eberhard, 1969; in males, see Appendix VI). The location and classification of brood within the

nest combs was mapped once during the monitoring period, so positions of cells with pupal caps could be known, and used with nest membership data (i.e. number of wasps on the nest) to represent nest characteristic measurements in analyses below. Brood classification consisted of egg, larva, pupa, empty cell, or parasitised cell (e.g. from interspecific Ichneumonoidea and Lepidoptera species).

Nests were checked once daily during daylight monitoring hours to identify newly emerged adult males. Dispersal rates were only recorded for males with known ages that were certain to have emerged on the monitored nest. Age and natal membership were assessed by identifying newly emergence males using the following criteria: shiny black eye colouration on emergence (Appendix VII); no wing wear from flying or fighting (Garcia & Noll, 2013); appearance of an unmarked wasp coincided with a broken-capped cell that once contained a pupa (i.e. a wasp had eclosed from the cell). After marking and measuring, newly emerged wasps were returned to the nest comb using forceps. We then observed the response of nestmates to newly marked males upon return for 5 min; if a recently marked male was ejected from the nest by nestmates or flew within 5 min, it was excluded from analysis as marking had likely interfered with natural dispersal. Using this 5 min check of 163 males marked in total, nine males were excluded. The age of marked males on dispersal from natal nests was recorded by identifying wasp IDs present on the nest (before marking new individuals). The age of dispersal for a male was determined as the first day that an individual was observed missing from the nest. If a marked male was observed on another nest, and dispersed again from this non-natal nest, the date from the natal nest was used as a true dispersal event.

Analysis: To investigate if age influenced natal dispersal, we ran a generalized linear mixed model (GZLMM) (binomial distribution, logit model), with the binary response variable of a dispersal event occurring or not. The fixed effect for the model was age, with the random effect of natal nest ID (intercept). The model accounted for multiple repeated measures on the same individuals by fitting time on the natal nest and wasp ID as random effect terms. Model fit was assessed by checking the residual deviance against the degrees of freedom. The *p* values were calculated using a likelihood ratio test of the model with and without the target factor. Variance of the random effect on the model is reported with standard deviation (SD). To describe dispersal timings in the population, a Kaplan-Meier estimation was used, reporting the dispersal probability with age as a survival plot (hazard ratio, HR).

Table 5.1. Characteristics of natal male nests for Question 1, with sample size and observations number for Questions 2 and 3. Sites used across the thesis: VV = Verdant Vale; EM = Eastern Main Rd; CT = Cumuto Tamana Rd; UWI = University Field Station of the University of the West Indies. Means to 1 d.p.

			Q1 Natural Dispersal Observation		Q1 Nest Comb Size and Number of Adults Present on Dispersal								Males Used for Questions…			
Nest ID	Year	Site	Number of Males Detected Dispersing	Departure Dates	Total Number of Cells	Number of Eggs	Number of Larvae	Number of Pupae	Number of Empty Cells	Number of Parasitised Cells	Mean Number of Adult ♀	Mean Number of Adult ♂	Q2 Number Collected	Q3 Number Collected	Q3 Number Dispersed	Q3 Departure Date
#01	2014	VV	1	20 Jun	160	13	0	1	146	0	6.0	1.0	-	-	-	-
#02	2014	VV	3	22–24 Jun	334	56	58	15	205	0	17.0	1.0	-	-	-	-
#03	2014	VV	1	25 Jun	104	32	36	14	22	0	10.0	0.0	-	-	-	-
#04	2014	VV	5	17–24 Jul	59	7	0	1	40	11	4.1	1.3	-	-	-	-
#05	2014	VV	2	29 Jun – 03 Jul	79	20	32	12	15	0	7.6	0.8	-	-	-	-
#06	2014	VV	4	13–14 Jul	146	30	37	26	35	18	10.5	2.5	-	-	-	-
#07	2014	VV	1	27 Jul	163	10	0	0	151	2	8.0	1.0	-	-	-	-
#08	2014	VV	6	25 Jun – 03 Jul	165	35	55	12	61	2	10.9	1.7	-	-	-	-
#09	2014	VV	2	24–28 Jul	171	38	34	10	88	1	6.8	0.3	-	-	-	-
#10	2014	VV	1	21 Jul	118	0	0	7	111	0	13.0	0.0	3	-	-	-
#11	2014	VV	10	25 Jun–03 Jul	165	4	1	6	154	0	5.9	2.8	-	-	-	-
#12	2014	EM	3	14–15 Jul	83	0	0	4	79	0	8.5	2.5	-	-	-	-
#13	2014	EM	2	02 Aug	152	11	2	18	121	0	7.0	2.0	-	-	-	-
#14	2014	EM	2	13–15 Jul	248	100	95	24	29	0	21.0	0.7	-	-	-	-
#15	2014	EM	5	18–20 Jul	70	6	0	2	62	0	5.0	1.3	-	-	-	-
#16	2015	VV	3	19–21 Jul	126	23	16	17	70	0	8.0	1.0	-	-	-	-
#17	2015	VV	4	22 July – 07 Aug	180	0	1	30	149	0	5.3	9.3	-	-	-	-
#18	2015	VV	10	22–29 Jul	117	0	0	5	112	0	3.3	4.6	-	-	-	-
#19	2015	CT	28	20 Aug–07 Sept	335	59	148	49	79	0	8.8	5.9	11	4	6	21–23 Sept
#20	2015	CT	30	29 Aug–07 Sept	484	131	157	131	65	0	26.0	26.9	46	11	5	21–23 Sept
#21	2015	CT	5	23 July, 06–08 Sept	128	51	56	18	3	0	5.7	3.7	2	7	11	21–22 Sept
#22	2015	EM	4	01–03 Sept	309	0	6	44	259	0	14.0	6.0	-	-	-	-
#23	2015	EM	18	31 Aug–02 Sept	-	-	-	-	-	-	17.0	9.7	-	-	-	-
#24	2015	EM	1	06 Sept	-	-	-	-	-	-	14.0	5.0	5	-	-	-
#25	2015	VV	1	23 Sept	-	-	-	-	-	-	16.7	7.0	3	10	-	-
#26	2015	VV	1	10 Sept	-	-	-	-	-	-	7.3	1.0	1	5	-	-
#27	2015	EM	1	09 Sept	-	-	-	-	-	-	-	-	-	-	4	23–24 Sept
#28	2014	VV	-	-	-	-	-	-	-	-	-	-	29	-	2	24–25 Sept
#29	2014	VV	-	-	-	-	-	-	-	-	-	-	10	-	-	-
#30	2014	VV	-	-	-	-	-	-	-	-	-	-	7	-	-	-
#31	2014	VV	-	-	-	-	-	-	-	-	-	-	16	-	-	-
#32	2015	CT	-	-	-	-	-	-	-	-	-	-	20	46	31	21–23 Sept
#33	2014	VV	-	-	-	-	-	-	-	-	-	-	3	-	-	-
#34	2014	VV	-	-	-	-	-	-	-	-	-	-	1	-	-	-
#35	2015	UWI	-	-	-	-	-	-	-	-	-	-	-	2	-	-
#36	2015	UWI	-	-	-	-	-	-	-	-	-	-	-	6	-	-
#37	2015	EM	-	-	-	-	-	-	-	-	-	-	-	-	2	23–25 Sept
				Mean ± SE	177.1 ± 22.3	28.5 ± 7.3	33.4 ± 10.0	20.3 ± 6.0	93.5 ± 13.8	1.5 ± 0.9	10.3 ± 1.1	3.8 ± 1.1				

As measured variables associated with nest characteristics (Table 5.1) were likely not to be independent (e.g. if larger nests have more brood and adults present), we reduced the dimensions of nest characteristics using a principle component analysis (PCA). First, a correlation coefficient matrix of nest characteristic variables was computed (variables: number of eggs, larvae, pupae, empty cells and parasitised cells in the nest; mean number of adult females present; and mean number of adult males present during dispersal periods), excluding variables from the subsequent PCA that had coefficients (r) < 0.3 (which would indicate no or non-linear relationships – Tabachnick & Fidell, 2007). Variables included in the PCA were centred and scaled. A Bartlett's sphericity test (Bartlett's test) was run to confirm statistical association of variables, and calculation of Kaiser-Meyer-Olkin (KMO) indexes performed to assess for sampling adequacy (KMOs > 0.5 – Child, 2006). We ran a PCA with included variables, listing components with eigenvalues > 1 (Girden & Kabacoff, 2010). A single component was generated (accounting for 76.94% of variance), and a factor score calculated (regression method) of included nest characteristic variables, for use as a single variable in subsequent Cox model analysis (referred to as 'active nest size').

To investigate what effects influenced age of natal dispersal, we ran multiple Cox models (Efron method for ties, describing hazard ratio as dispersal rate). First, we investigated the fixed effects of wing length, active nest size, number of empty cells (excluded PCA variable), number of parasitised cells (excluded PCA variable), and whether a male received aggression upon returning to the nest after being marked; clustered by location and year. Second, we reran the first model exchanging the fixed effect of active nest size with one of its original components 'mean number of females present' (reporting the test value of the single effect, and if variables in the first model remained significant or not). The second model was created as the mean number of females present might directly influence male dispersal, because female nestmates forage and bring food to the nest (or potentially aggress newly emerged males). Lastly, we investigated whether dispersal between the three sites varied, by running a series of Cox models pairing each site as a fixed effect clustered by nest and year (i.e. site VV to EM, site VV to CT, and site EM to CT). Regarding the assumptions of Cox modelling: though nests likely varied throughout the observation period in their activity (e.g. number of females, cells, etc.), we assume proportional hazards are relatively constant and use mean values when possible to capture fluctuation in nest activity.

5.3.3. Question 2: Do males delay dispersal until they are sexually mature?

To assess sexual maturation, we measured the sizes of different male reproductive organs in males of different ages and dispersal states. Typical indicators of sexual maturation in male wasps include reduction in the testes area (from degradation, as spermatozoa production ceases) and an increasement in fluid area inside both the seminal vesicles and accessory glands (Moors et al., 2009). We tested whether size and fluid content of these reproductive organs changed between ages of natal males, and whether organ measurements in non-natal (dispersed but found on a non-natal nests) and truly dispersed (collected off-nests) males were significantly different in comparison to natal nesting males.

Sample collection: A total of 169 males were collected for dissection at at sites VV, EM, and CT between June and September in 2014 and 2015 (Table 5.1). We defined three types of male: 'natal' – a male still on his natal nest; 'dispersed non-natal' – a dispersed male caught on another nest (i.e. not his natal); and 'dispersed non-nesting' – a male caught around the site on vegetation etc., not on any nests. Sample sizes included 144 natal males from 11 nests with known ages through marking: 42 newly emerged males (zero-day-old males collected on day of emergence); 38 one-day-old males; 35 two-days-old males; 21 three-days-old males; three four-days-old males; two five-days-old males; and three natal males of undetermined age (included in analysis of natal to dispersed males). Additionally, we collected 13 dispersed non-natal males on six nests, and 12 dispersed non-nesting males caught around the sites. All males were stored in 70% ethanol at -20°C upon collection.

Dissections: We conducted male dissections in phosphate-buffered saline, using a Leica M165 C stereo microscope with a Leica IC80 HD digital camera attachment. Dissections were conducted by cutting a rectangular section in the ventral side of the abdomen and peeling back tergites, removing the testes, and the pair of seminal vesicles with accessory glands. Removed organs were prepared on a slide, and photographs taken. Using ImageJ 1.51j8, two-dimension measurements were taken of the area around the outer layer of the testes (mm^2 to 2 d.p.), and the area of the fluid inside the seminal vesicles and accessory glands separately (mm^2 to 2 d.p.) (Figure 5.1). Fluid area of the seminal vesicles and accessory glands is reported as the mean area between paired organs respectively, as the simultaneous function of ejaculation is not known. In analysis, fluid area is referred in relation to mSV (mean seminal vesicle) and mAG (mean accessory gland). Scoring and measurements were conduct blind to the age of the individual.

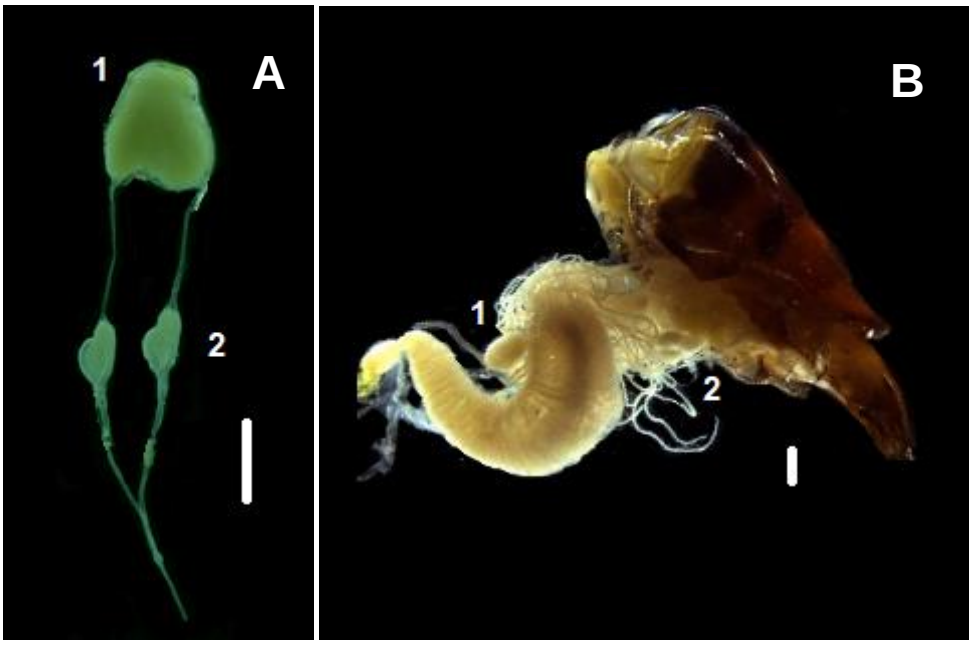


Figure 5.1. Reproductive tract of *P. lanio* male: 1 = testes area; 2 = pair of seminal vesicles and accessory glands; white bar = 0.5 mm scale. (A) Dissected reproductive tract (from top to bottom) testes connected into seminal vesicles (which have the accessory glands joined below them), seminal vesicles and accessory glands connect and follow into the aedeagus; (B) reproductive tract 'nested' within malpighian tubules.

Analysis: First, to determine whether body size was a potential cofounding variable, we assessed if wing length was correlated with early reproductive development (testes area, mSV and mAG fluid area) in zero-day-old males, using Pearson's product-moment correlations (Pearson's correlation). Variables were plotted with histograms and Q–Q plots to assess deviations from a normal distribution. We removed three outliers for mAG to achieve a normal distribution.

Second, to investigate if male reproductive organ maturation was related to age on natal nests, we ran three separate general linear mixed models (GLMM) with response variables of testes area, mSV or mAG fluid area. Response variables were plotted with histograms and Q–Q plots to assess deviations from a normal distribution. We removed two outlier data points for testes area to achieve a normal distribution. The fixed effects for models consisted of age, site location, wing length and year. The random effect was natal nest ID (intercept). Model fits were assessed by checking a residual vs fitted value plot. The *p* values were calculated using a likelihood ratio test of the model with and without the target factor. Variance of the random effect on the models is reported with SD. If the random effect had a variance and SD of 0.00,

the effect was dropped, and the model retested with the above variables using a general linear model (GLM).

Finally, to investigate difference in male reproductive organ maturation between natal, dispersed non-natal, and dispersed non-nesting males, we ran three separate GLMs with response variables of testes area, mSV or mAG fluid area. Response variables were plotted with histograms and Q–Q plots, to assess deviations from a normal distribution. We removed three outliers for mSV, and one for mAG, to achieve a normal distribution. The fixed effects for models consisted of nesting state (i.e. natal, dispersed non-natal or dispersed non-nesting), site location, wing length and year. Model fits were assessed by checking a residual vs fitted value plot. The *p* values were calculated using a likelihood ratio test of the model with and without the target factor. We performed a post-hoc Tukey test on the multilevel variable of nesting state (single-step method for *p* value adjustment).

5.3.4. Question 3: Does JH regulate male natal dispersal?

To determine the influence of JH on the timing of male dispersal from the natal nest, we first determined the appropriate dose of JH analogue methoprene to use in experiments on *P. lanio* males. Then we used topical applications of methoprene on newly emerged adult males, and recorded subsequent departure rates. Finally, we repeated applications on captive males to assess the effects of methoprene application on reproductive maturation.

Lab-based determination of a suitable dose of methoprene: Methoprene is a readily available analogue of JH that is commonly used in insect-based experiments of this type, including in *Polistes spp.* (Giray et al., 2005; Shorter & Tibbets, 2009). However, to our knowledge no published data exist for application of methoprene on males in this genus, and therefore we first needed to determine a suitable non-lethal dose to use in the experiments. We did this by applying a range of dosages to males, and comparing survivorship. Seventy-two natal males (of approx. one- to seven-days-old) were collected alive from seven nests at sites VV, CT and UWI in September 2015 (Table 5.1). Topical applications of 1 µl methoprene/acetone treatments were made to the dorsal side of the thorax using a micro-syringe, with three dosages of methoprene (µg) in acetone (µl) – 0 µg/µl, 250 µg/µl and 500 µg/µl – and a blank control (with dummy handling). Treatments had 18 males per group. The first dose was chosen randomly; thereafter, dose treatments were applied sequentially for males collected within the same nesting group. The choice of dosage volume was based on studies of methoprene applications in *A. mellifera* males (Sasaki et al., 2012). Males were held by forceps after application until the thorax appeared dry, and wings marked for individual identification with Uni POSCA markers. Males from the same nest were then housed in 25×15×15 cm plastic enclosures, with separate enclosures for each nest group. These enclosures were exposed to field-realistic conditions, but sheltered from direct sunlight, rain and ant predation. All males

had *ad libitum* access to food, in the form of a cut piece of starch mango *Mangifera indica*, and water. Mortality rates were recorded every 24 h for 10 days from initial application.

A Cox model (Efron method for ties) was used to determine whether mortality rates between dosages, clustered by nest, significantly differed from that of the blank control treatment. Proportional hazards were assumed, as housing conditions did not change throughout the trial.

Field-based experiment on the effects of JH on timing of male dispersal: To observe the effect of methoprene on dispersal behaviour, 61 newly emerged males (zero-day-old individuals) from seven nests at sites VV, EM and CT were selected for experimental treatment in September 2015 (Table 5.1). Each male was marked and treated during daylight hours using first-order randomisation and sequential assignment within nests, with a 1 µl topical application of either 0 µg/µl (control, 30 males) or 250 µg/µl (methoprene, 31 males) methoprene in acetone, as per the lab-based trial above. Treated males were then returned to their natal nests and observed for 5 min (there were no instances of immediate departure). Nests were then monitored 24 and 48 h after treatment to determine timing of dispersal. Dispersal day was recorded as the date of the first census where a male was permanently absent from the natal nest.

To test whether methoprene application induced early departure of males from their natal nest, a Cox model (Efron method for ties, describing hazard ratio as dispersal rate) was used to determine whether dispersal rate of those treated with methoprene differed from that of the control treatment, clustered by nest. Proportional hazards were assumed, as dispersal was recorded from only the natal nest.

Effect of JH on reproductive maturation: To ascertain whether methoprene promotes early reproductive development, 19 males were collected alive from six nests at sites VV, CT and UWI in September 2015 (Table 5.1). Each male was marked and treated using first-order randomisation and sequential assignment within nests with a 1 µl topical application (protocol as above) of either 0 µg/µl (control, n=11 males) or 250 µg/µl (methoprene, n=8 males) methoprene in acetone. These males were then housed in plastic enclosures (as above). After 48 h, males were collected and stored in 70% ethanol at -20°C. Reproductive organ measurements were taken (protocol Question 2). There was no male mortality observed in enclosures within the 48 h period.

To test whether methoprene applications accelerated maturation with the 48 h, we ran three separate GLMMs with response variables of testes area, mSV or mAG fluid area. Response variables were plotted with histograms and Q–Q plots, to assess deviations from a normal distribution. As sample size was small, we did not remove outliers. The fixed effects for models

consisted of methoprene treatment (i.e. natal, non-natal or dispersed off-nest), with the random effect of natal nest ID (intercept). Model fits were assessed by checking a residual vs fitted value plot. The *p* values were calculated using a likelihood ratio test of the model with and without the target factor. Variance of the random effect on the models is reported with SD. If the random effect had a variance and SD of 0.00, the effect was dropped, and the model retested with the above variables using a GLM.

5.3.5. Statistical analyses

Analyses were performed in R 3.3.3 (R Core Team, 2017), using packages 'car' (Fox & Weisberg, 2011), 'ggpubr' (Kassambara, 2017), 'Hmisc' (Harrell Jr et al. 2017), 'lme4' (Bates et al., 2015), 'lmerTest' (Kuznetsova et al., 2016), 'multcomp' (Hothorn et al., 2008), 'RLRsim' (Scheipl et al., 2008) and 'survival' (Therneau & Grambsch, 2000). All analyses were tested at a significance of $\alpha = 0.05$, with averages reported as median with IQR or mean ± SE when applicable. In reporting data averages (in text or plotted graphs), we include outliers that were removed from statistical analysis.

5.4. Results

5.4.1. Question 1: Is male dispersal from the natal nest age-determined?

All males eventually dispersed from their natal nests. Male dispersal age from natal nests ranged from one to seven days old in the population (27 nests, 154 males), with the median age of dispersal being three-days-old (IQR = 2.0). Male age on dispersal had a significant positive effect with dispersal events occurring (GZLMM: $\chi^2_1 = 33.77$, $p < 0.001$). Dispersal probability increased between each age category, with chance to disperse between ages ranging from 75 to 98% (Figure 5.2). There was significant variance in the model attributed to the random effect of natal nest origin (variance = 0.38, SD = 0.62; $\chi^2 = 5.18$, $p = 0.023$).

In 13% of dispersal events from the natal nest (20 out of 155 events), males appeared on non-natal foundress and post-emergence nests. These non-natal nests ranged from 0.5 to 16.5 m away from their natal nests (skewed towards lower dispersal distances; Figure 5.3), with males appearing either on the same day or 1–2 days after dispersal. Non-natal males were a median age of two-days-old (IQR = 1.75), and stayed on non-natal nests for 1–2 days. Males always eventually left these non-natal nests, and in only two instances did a male appear on a second non-natal nest (these were post-emergence nests, 5.0 and 10.0 m away from the first non-natal nest), where the males remained for 1 day each before becoming undetected (presumably dispersed).

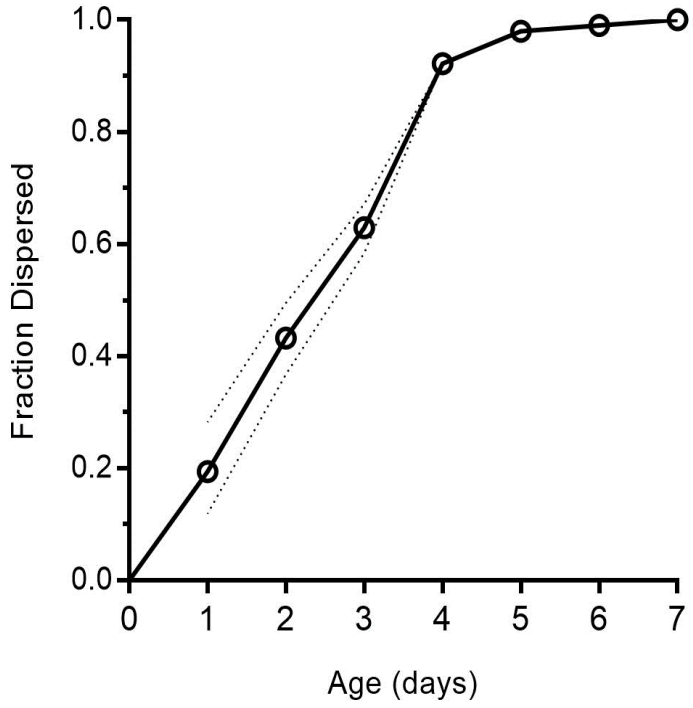


Figure 5.2. Question 1: Male dispersal rates from natal nests in the population (lower & upper 95% CI) by age (Kaplan-Meier estimation).

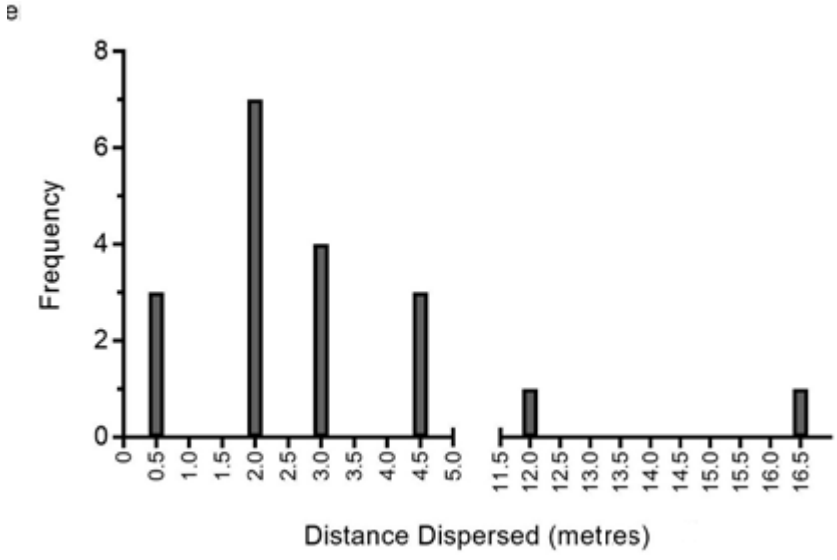


Figure 5.3. Question 1: Distances dispersed (metres, approximately to .5 of integer) in male dispersal events onto non-natal nests.

Variables associated with natal nest characteristics were reduced to a single term using PCA. Using a correlation coefficient matrix (Figure 5.4), variables included (r > 0.3) in a subsequent PCA were: number of eggs, larvae, pupae in the nest; and mean number of adult females and males present (separately) during male dispersal periods. Included variables had a mean correlation coefficient to each other of r = 0.71 ± 0.04. We excluded the following variables (r < 0.3): number of empty cells (mean correlation coefficient to all other variables, r = -0.14 + 0.08); and number of parasitised cells (r = -0.12 + 0.03). Bartlett's test confirmed statistical association between included variables (χ^2_{10} = 115.461, p <0.001). Overall KMO index of included variables = 0.61, suggesting adequate sampling for the PCA (KMO > 0.5) (individual KMO indexes: eggs = 0.65; larvae = 0.60; pupae = 0.60; adult females = 0.62; adult males = 0.56). A PCA of these variables generated a single component which accounted for 76.94% of the variance (eigenvalue = 3.844, communalities range: 0.656–0.871, individual component loadings: eggs = 0.907; larvae = 0.892; pupae = 0.933; adult females = 0.837; adult males = 0.811). Based on the grouping of this single component, a factor score of included variables was calculated, and used in the GZLM analysis below ('active nest size')

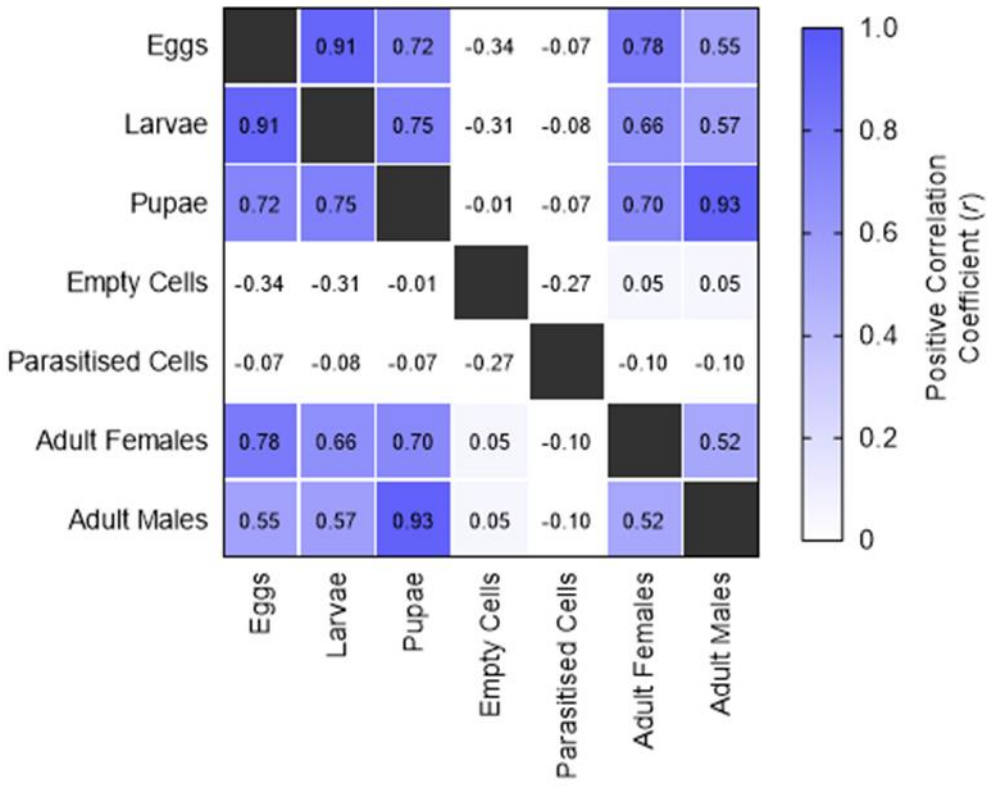


Figure 5.4. Question 1: Correlation coefficients matrix (mirrored) between natal nest characteristic variables, showing positive correlations between all nest variables except for number of empty and parasite cells (in relation to natural male dispersal observations).

Analysis of body size and nest-related variables on male dispersal age revealed no influence on the age of departure event. No significant effects on dispersal age were detected for male wing length (Cox model: Wald χ^2_1 = -0.090, HR = 0.99, lower 95% CI = 0.82, upper 95% CI = 1.20, p = 0.928; mean wing length = 18.24 ± 0.09 mm), active nest size (χ^2_1 = 0.547, HR = 1.04, lower 95% CI = 0.89, upper 95% CI = 1.22, p = 0.585), number of empty cells (χ^2_1 = -0.396, HR = 1.00, lower 95% CI = 1.00, upper 95% CI = 1.00, p = 0.692), number of parasitised cells (χ^2_1 = 0.461, HR = 1.01, lower 95% CI = 0.97, upper 95% CI = 1.05, p = 0.645), or aggression received after marking (χ^2_1 = 1.617, HR = 1.422, lower 95% CI = 0.93, upper 95% CI = 2.18, p = 0.106; 8% of non-immediately departing males received aggression). The mean number of females present during departure periods, analysed separately, also had no significant effect on male dispersal age (χ^2_1 = -0.045, HR = 1.00, lower 95% CI = 0.97, upper 95% CI = 1.03, p = 0.964; for all other variables listed above $p > 0.05$). Comparisons of male dispersal ages between sites also revealed no differences in departure age. Male dispersal rate was not significantly different between site VV and EM (Site EM: χ^2_1 = -0.425, HR = 0.88, lower 95% CI = 0.48, upper 95% CI = 1.61, p = 0.671), site VV and CT (Site VV: χ^2_1 = -1.485, HR = 0.76, lower 95% CI = 0.53, upper 95% CI = 1.09, p = 0.138), and site EM and CT (Site CT: χ^2_1 = 1.396, HR = 1.54, lower 95% CI = 0.84, upper 95% CI = 2.83, p = 0.163). Median male dispersal age was four-days-old (IQR = 2.0) at EM, three-days-old (IQR = 2.0) at VV, and two-days-old (IQR = 2.0) at CT.

5.4.2. Question 2: Do males delay dispersal until they are sexually mature?

No significant evidence was found that wing length significantly correlated with initial reproductive development in newly emerged zero-day-old males (10 nests, 42 males) (Pearson's correlations: testes area, r_{41} = 0.09, p = 0.586; mSV fluid area, r_{41} = 0.12, p = 0.439; mAG fluid area, r_{38} = 0.04, p = 0.809). Mean measurements for newly emerged zero-day-old males were: wing length, 18.05 ± 0.13 mm; testes area, 1.84 ± 0.07 mm^2; mSV fluid area, 0.07 ± < 0.01 mm^2; mAG fluid area, 0.09 ± < 0.01 mm^2 (Figure 5.5).

Male age was associated with reproductive maturity on the natal nest, with older males being more mature than younger males (12 nests, 144 males). There was a significant negative relationship of testes area with age (GLMM: χ^2_1 = 33.05, p <0.001). No significant variation in testes area was detected between sites (χ^2_2 = 0.49, p = 0.783), in relation to wing length (χ^2_1 = 2.78, p = 0.096; mean wing length = 18.00 ± 0.07 mm), or between the two years (χ^2_1 = 0.06, p = 0.800). There was no significant variation in testes area due to the random effect of natal nest origin (variance = 0.01, SD = 0.08; χ^2 = 0.16, p = 0.137). There was a significant positive relationship of mSV fluid with age (GLM: F_1 = 29.81, p <0.001). No significant effect with mSV fluid area was detected between sites (F_2 = 0.07, p = 0.931), to wing length (F_1 = 2.25, p = 0.136), or between the two years (F_1 = 0.30, p = 0.585). No effect with the random effect of

natal nest origin and mSV fluid area was detected (variance = 0.00, SD = 0.00; random effect dropped from the model). There was a significant positive relationship between mAG fluid and age (GLM: F_1 = 33.95, p <0.001). No significant effect with mAG fluid area was detected between sites (F_2 = 1.31, p = 0.273), to wing length (F_1 = 2.05, p = 0.155), or between the two years (F_1 = 1.03, p = 0.312). No effect with the random effect of natal nest origin and mAG fluid area was detected (variance = 0.00, SD = 0.00; random effect dropped from the model) (Figure 5.6).

Signs of greater reproductive maturity were detected in dispersed non-nesting males, compared to males still nesting (both natal and dispersed non-natal) (169 males). Nesting state had a significant effect with testes area (GLM: F_2 = 13.59, p <0.001). Dispersed non-nesting males had significantly smaller testes than natal males (Tukey: t-value = -5.12, p < 0.001), and dispersed non-natal males (t-value = -2.77, p = 0.017). Testes area was not significantly different between natal and dispersed non-natal males (t-value = -1.52, p = 0.276). No significant effect with testes area was detected between sites (F_2 = 0.261, p = 0.771), to wing length (F_1 = 0.833, p = 0.363; mean wing length = 18.08 ± 0.07 mm), or between the two years (F_1 = 0.149, p = 0.700). Nesting state had a significant effect with mSV fluid area (F_2 = 22.58, p < 0.001). Dispersed non-nesting males had significantly more mSV fluid than natal males (Tukey: t-value = 6.63, p < 0.001), and dispersed non-natal males (t-value = 4.12, p < 0.001). mSV fluid area was not significantly different between natal and dispersed non-natal males (t-value = 1.81, p = 0.163). No significant effect with mSV fluid area was detected between sites (F_2 = 0.02, p = 0.980), to wing length (F_1 = 2.72, p = 0.101), or between the two years (F_1 = 0.11, p = 0.741). Nesting state had a significant effect with mAG fluid area (GLM: F_2 = 16.42, p < 0.001). Dispersed non-nesting males had significantly more mAG fluid than natal males (Tukey: t-value = 5.66, p < 0.001), and dispersed non-natal males (t-value = 3.34, p = 0.003). mAG fluid area was not significantly different between natal and dispersed non-natal males (t-value = 1.46, p = 0.306). No significant effect with mAG fluid area was detected between sites (F_2 = 0.81, p = 0.446), wing length (F_1 = 2.26, p = 0.135), or between the two years of study (F_1 = 0.58, p = 0.448) (Figure 5.7).

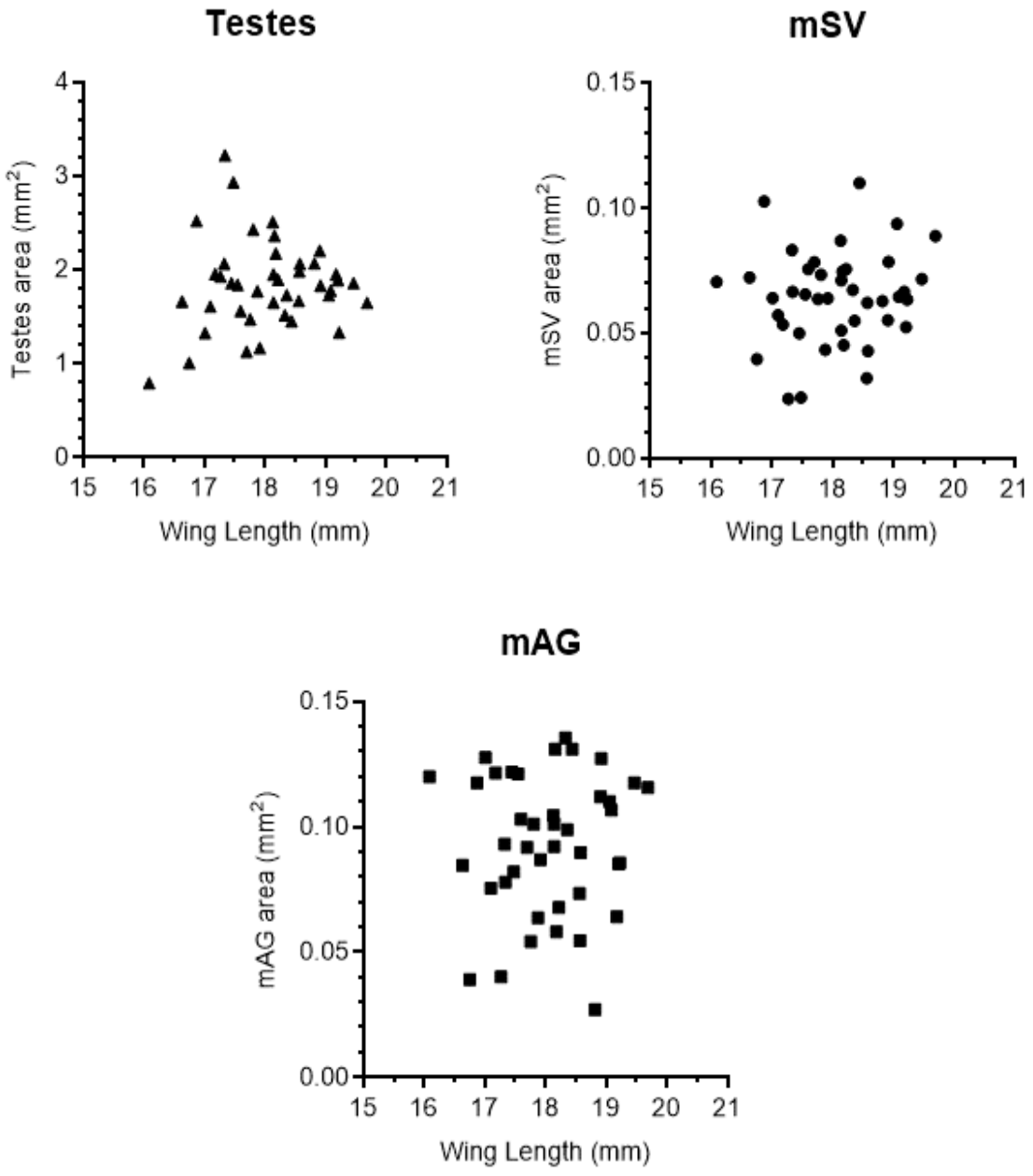


Figure 5.5. Question 2: Relationship between male wing length with that of testes area, mSV fluid area, and mAG fluid area in newly emerged zero-day-old adult males. Pearson's correlations not significant, α = 0.05.

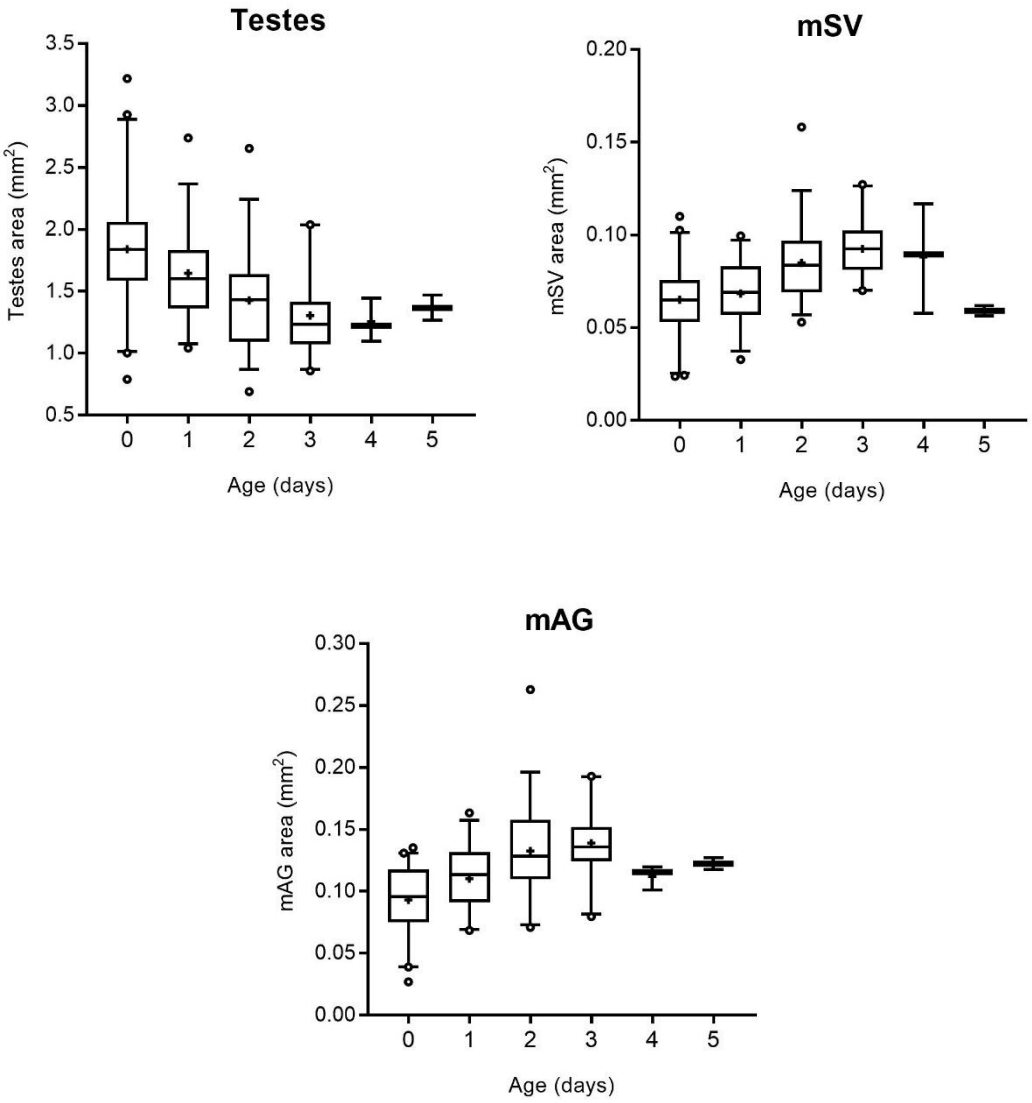


Figure 5.6. Question 2: Age has a significant negative relationship with testes area (GLMM, $p < 0.001$), and a positive relationship with mSV fluid area (GLM, $p < 0.001$) and mAG fluid area ($p < 0.001$). Box (25th to 75th) and whisker (5th to 95th) plots with mean centre points of male testes area, mSV fluid area, and mAG fluid area by age on natal nests. Points on graph indicate outliers.

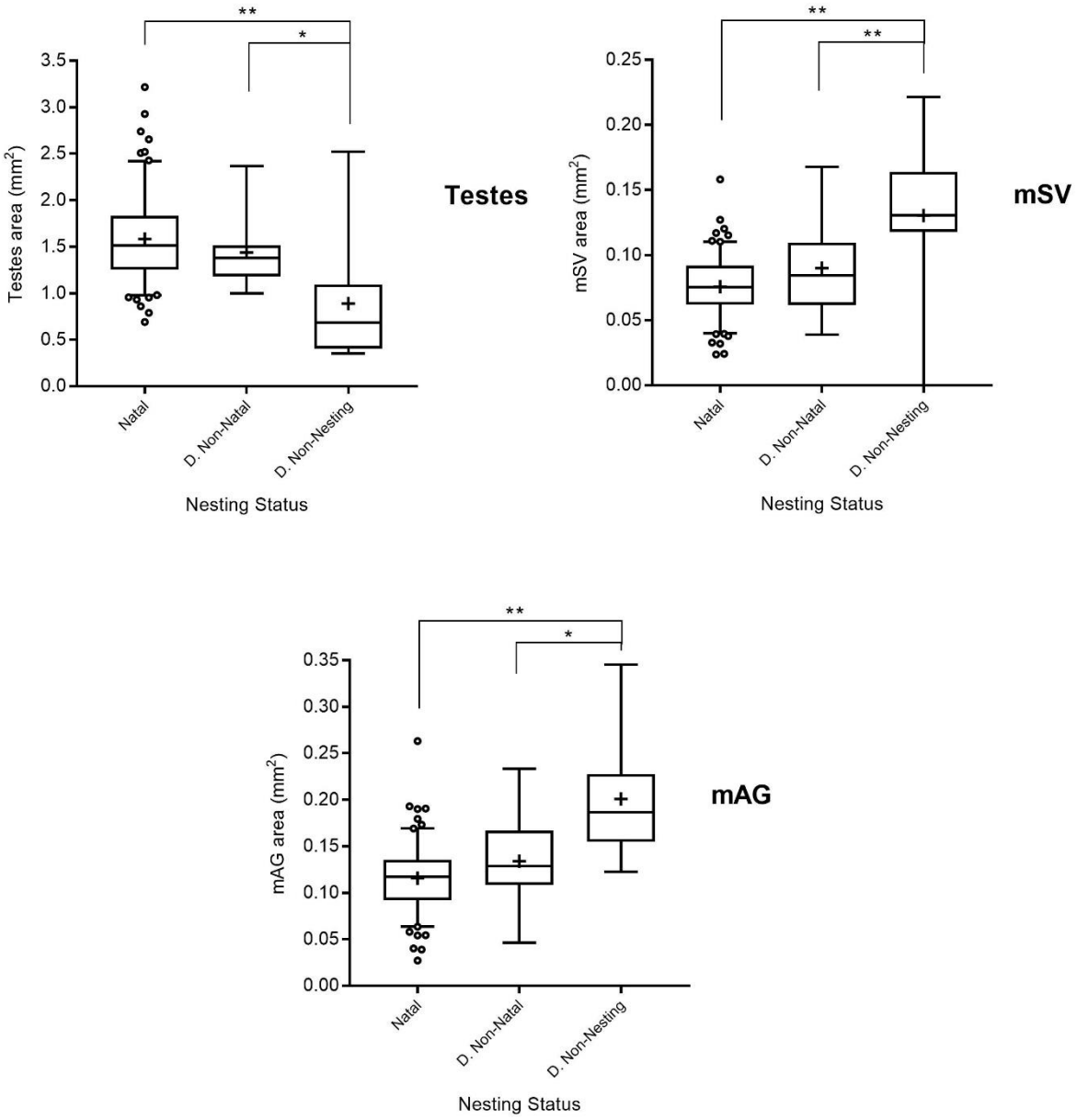


Figure 5.7. Question 2: Significance difference (Tukey: * $p < 0.05$; ** $p < 0.001$) between signs of reproductive maturation in dispersal non-nesting males to natal and dispersed non-natal nesting males; no significant difference between natal and dispersed non-natal groups. Box (25th to 75th) and whisker (5th to 95th) plots with mean centre points of male testes area, mSV fluid area, and mAG fluid area by nesting status (either: natal, dispersed non-natal, or dispersed non-nesting). Points on graph indicate outliers.

5.4.3. Question 3: Does JH regulate male natal dispersal?

In lab-based trials to determine the appropriate levels of methoprene (72 males), males treated with 500 µg/µl methoprene had significantly higher mortality rates compared to blank controls (Cox model: Wald $\chi^2_3 = 3.544$, HR = 6.91, lower 95% CI = 2.37, upper 95% CI = 20.14, $p < 0.001$), confirming that the treatment had been successfully absorbed through the carapace. Males treated with 500 µg/µl methoprene were seven times more at risk of mortality than blank control treated males. Survivorship of males treated with 0 µg/µl (Wald $\chi^2_3 = 1.508$, HR = 1.87, lower 95% CI = 0.83, upper 95% CI = 4.31, $p = 0.132$) and 250 µg/µl methoprene (Wald $\chi^2_3 = -0.595$, HR = 0.66, lower 95% CI = 0.17, upper 95% CI = 2.61, $p = 0.552$) was not significantly different from blank control treated males. Thus, treatment with 250 µg/µl methoprene was considered appropriate for use in further methoprene experiments (Figure 5.8).

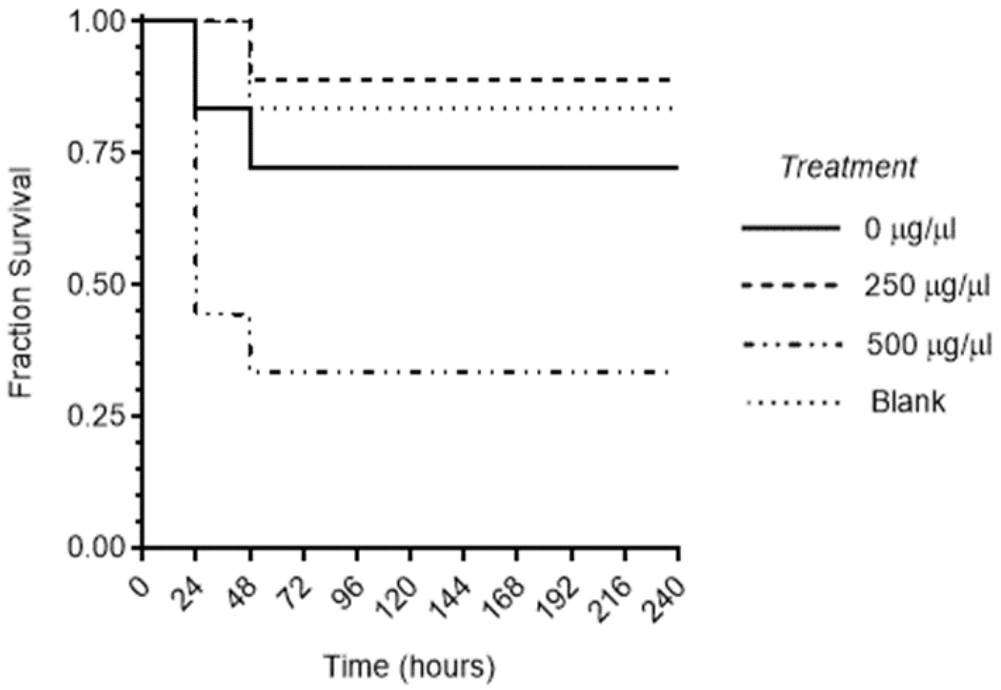


Figure 5.8. Question 3: Significant (Cox model) higher morality hazard rates in males treated with 500 µg/µl to dummy control males ($p < 0.001$). Treatments 0 µg/µl and 250 µg/µl not significant to blank control group. Survival analysis of males treated with 0 µg/µl, 250 µg/µl, or 500 µg/µl of methoprene in acetone, and a blank dummy control, to select a suitable dosage for further testing.

Treatment of zero-day-old newly emerged males with methoprene on the natal nest (7 nests, 61 males) induced early dispersal. Males treated with 250 µg/µl methoprene were three times more likely to disperse earlier than 0 µg/µl control males (Cox model: Wald $\chi^2_1 = 3.823$, HR = 3.16, lower 95% CI = 1.76, upper 95% CI = 5.80, $p < 0.001$) (Figure 5.9).

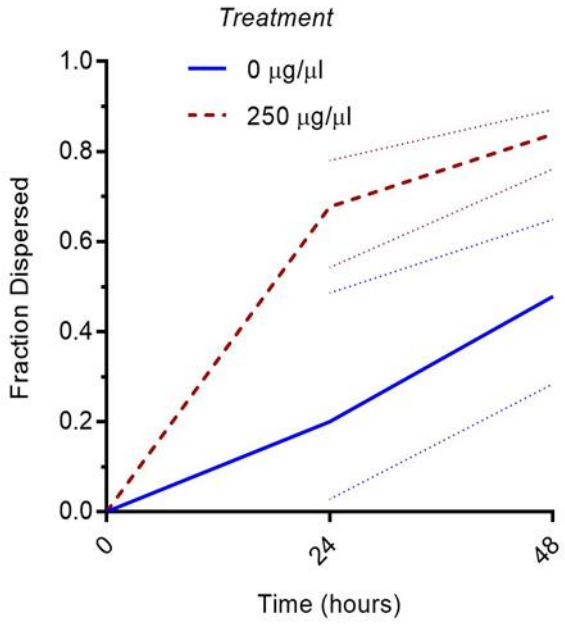


Figure 5.9. Question 3: Significant (Cox model) earlier dispersal rates in males treated with methoprene ($p < 0.001$). Male natal dispersal rate (lower & upper 95% CI) in newly emerged males treated with 0 µg/µl or 250 µg/µl of methoprene in acetone.

In further lab-based trials, males treated with 250 µg/µl methoprene at zero days of age showed earlier signs of sexual maturity relative to 0 µg/µl control males of the same age, 48 h after application from adult emergence (19 males). mAG fluid area of males treated with 250 µg/µl methoprene was significant greater than 0 µg/µl control males (GLM: $F_1 = 4.60$, $p = 0.047$; random effect of natal nest origin – variance = 0.00, SD = 0.00, effect dropped from the model). However, there was no significant difference in mSV fluid area (GLM: $F_1 = 0.47$, $p = 0.502$; random effect of natal nest origin – variance = 0.00, SD = 0.00, effect dropped from the model) and testes area (GLMM: $\chi^2_1 = 0.04$, $p = 0.851$; random effect of natal nest origin – variance = 0.11, SD = 0.33, no significant nest variance $\chi^2 = 0.68$, $p = 0.136$) (Figure 5.10).

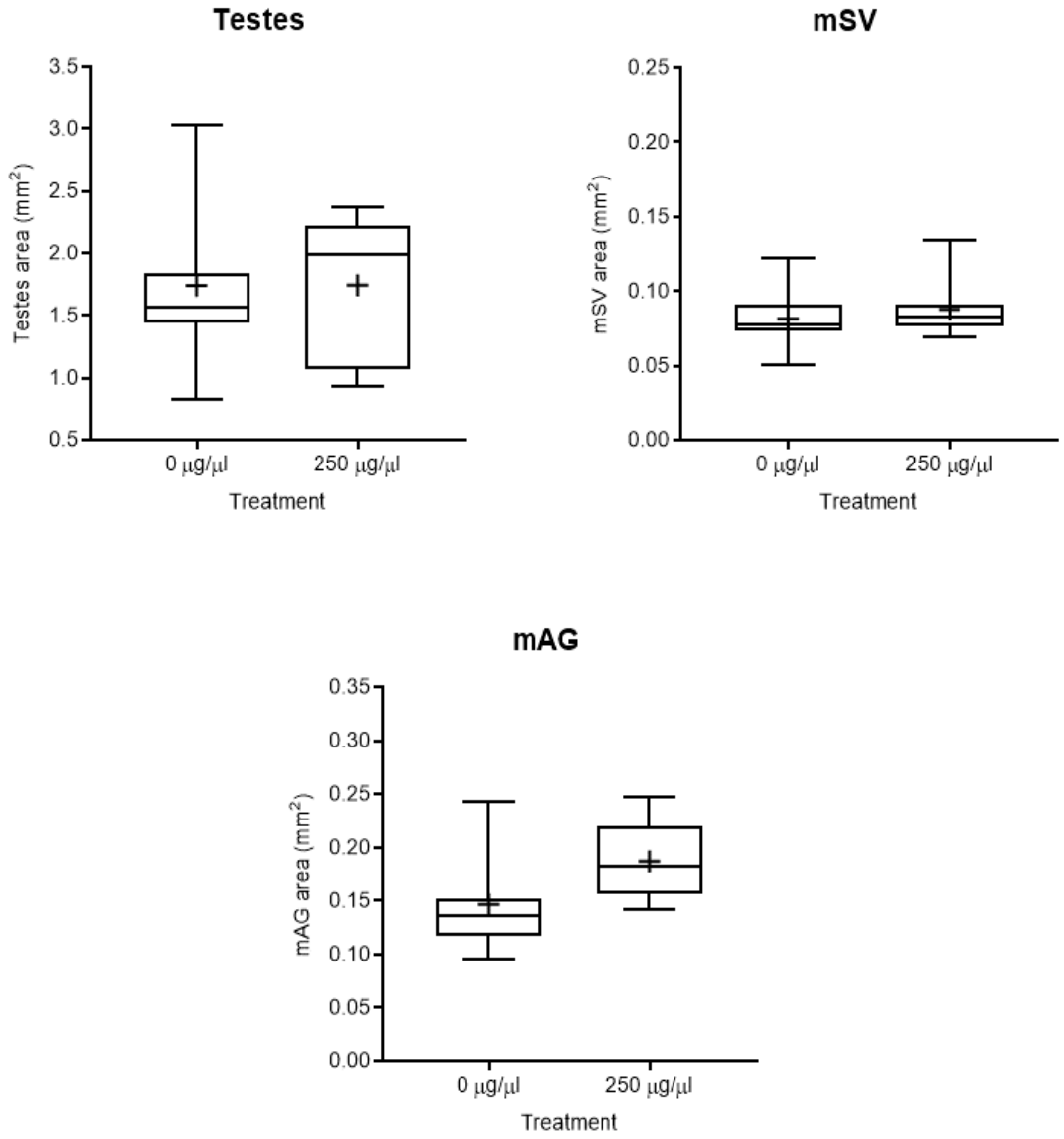


Figure 5.10. Question 3: mAG fluid area sig. increase (GLM, $p < 0.05$) in methoprene treated males. Box (25th to 75th) and whisker (5th to 95th) plots with mean center points of male testes area, mSV fluid area, and mAG fluid area by treatment (0 µg/µl or 250 µg/µl of methoprene in acetone) after two days from emergence and application.

5.5. Discussion

In the social Hymenoptera, delayed male dispersal from the natal nest is likely to correlate with a period of sexual immaturity after adult emergence. Here, we provide evidence that males of the simple eusocial paper wasp *P. lanio* delay dispersal from the natal nest, and that dispersed males tend to be sexually mature relative to both natal and non-natal nesting males. Further, experimental treatment of the JH analogue methoprene induces precocious dispersal, and increased fluid production of the accessory glands (a sign of sexual maturity). Sexual maturation in adult insects is widely regulated by JH, and JH is likely to be both a behavioural and gonadotropic regulator of dispersal in *P. lanio* males.

Male dispersal was predicted by age. Age-determined dispersal of males indicates that male dispersal is strongly regulated internally by chronology. Individuals across taxa typically disperse from natal groups within a fixed age range, and this may correlate with sexual maturation and/or first breeding attempts (Blair, 1953; Martín & Bucher, 1993; Künkele & von Holst, 1996; Van Horn et al., 2003; González et al., 2006; Fernandez-Duque, 2009). Males in our *P. lanio* population dispersed from the natal nest at one- to seven-days-old, similar to male dispersal age ranges found in other *Polistes* and closely related wasp species, irrespective of temperate or tropical environments (*Polistes ferreri*, zero- to five-days-old; *Polistes jokahamae*, zero- to six-days-old; *Polistes major*, zero- to thirteen-days-old; *Ropalidia marginata*, zero- to eight-days-old; *Ropalidia cyathiformis*, mean dispersal 19 days-old – Gadagkar & Joshi, 1984; Cameron, 1986; Makino, 1993; Sinzato et al., 2003; Sen & Gadagkar, 2006; Sen & Gadagkar, 2011). However, in a Brazilian population of *P. lanio*, male dispersal age ranged from zero to 31 days-old (Giannotti, 2004). What differences may exist between Trinidadian and Brazilian populations to account for such wide dispersal age ranges is unknown.

Male Hymenoptera in comparison to females have much shorter lifespans, adopting a strategy of 'live-hard, die young' (Heinze, 2016). Given that there is likely an interconnected trade-off between lifespan, dispersal and reproductive effort, individuals that disperse from natal locations may have increased reproductive output at the cost of shortened lifespans (Cotto et al., 2015). If the life history strategy of males is to disperse and mate as soon as possible, it seems contradictory that adult males do delay departure.

Dispersal is not always age-determined, but can be facultative and responsive to the environment, with individuals using decision thresholds based on the surrounding abiotic, biotic, and social environment to judge when dispersal is optimal (Bowler & Benton, 2005; Clobert et al., 2009). Though most environmental variables directly recorded in this study, such as nest size and location, did not have an influence on male natal dispersal rates; significant variance was detected between nests (as a random effect – Question 1). It could be

argued that variation in the timing of dispersal therefore exists independent of age effects. What precise variables are associated with this variance is unknown. Individuals may use decision-based thresholds in conjunction with chronological age, to judge when dispersal is optimal. For example, male dispersal in yellow baboons *Papio cynocephalus* increases if reproductive success in a group is low (Alberts & Altmann, 1995), and natal dispersal rates in offspring of subsocial black lace-weaver spiders *Amaurobius ferox* decrease with the increased availability of prey items on the web (Kim, 2000). There could be additional factors, such as female foraging rate, nesting density, number of receptive females, or lek activity in the surrounding population that influence male dispersal rates. Males also seem to have the opportunity to mature and disperse from non-natal nests (i.e. natal disperse, then disperse a second time). It is not known whether dispersed non-natal males are related to the non-natal nests they visit; if so, then non-natal males might gain protection from predation and nutrition from foragers. Alternatively, if these non-natal males are un-related to the nests they visit, they might be either sneak/low cost intruders. Nest drifting is a common behaviour in females of tropical *Polistes*, whereby workers split time on natal and non-natal nests (Sumner et al., 2007). This non-natal behaviour of males differs from female drifting, in that males never return to either natal or non-natal nests. However, it is likely that the mechanisms permitting females to move between nests predisposes males to do the same.

Another mechanism that could regulate male dispersal is harassment by females. There are several reasons why it is in the females' interests to exclude males as soon as possible from the nest. First, males are generally thought to be a drain on the group resources (Trivers & Hare, 1976). Second, there may be selection for females to exclude males to avoid inbreeding with siblings on the natal nest (Pusey & Wolf, 1996). Males are typically less aggressive and easily dominated by females in *Polistes* (Polak, 1992), hence if male age and maturation correlated with a behavioural or odour cue to females, their sisters could physically eject them from the natal nest. However, we found no evidence that male dispersal was influenced by our measures of the female social environment, either in number of females present or aggression received upon marking return. Future studies could employ more precise measurements, such as aggression received by males as they age on the natal nest.

Newly emerged males are reproductively immature, and they develop sexually during their time on the nest. Dissections suggested that males mature with age on the nest, and those that disperse (caught off-nests) show significant signs of sexual maturation in comparison to nesting males. It may be that males remain on the nest for a time as they sexually mature. Successful acts of copulation indicate full maturation, but we did not observe this behaviour, and can therefore only infer if a male is more or less mature than another individual. Age at full maturation varies across species in the Hymenopteran, but is usually greater than seven-days-old in social bees and wasps (reviewed, Table 5.2). As male dispersal age in our *P. lanio* population fell below or on seven-days-old, it may be that dispersed males whilst more mature

than at emergence, are not fully mature. There may be selection for males to disperse just before reaching full maturation, in order to reduce chances of inbreeding (Pusey & Wolf, 1996).

Table 5.2. Minimum age ranges of full maturation in male Hymenoptera. Notes: 1 - defined as complete sperm transfer into seminal vesicles; 2 - only in 10% of trials did males age zero- to one-days-old mate, compared to 80% with males older than two-days-old.

Level of Socality	Family	Species	Definition of Maturity	Minimum Age	Reference
Solitary Parasitoid	Braconidae	*Fopius vandenboschi*	*Successful Mating*	Immediate	Ramadan et al., (1991)
		Fopius arisanus	*Successful Mating & Complete Reproductive Maturation*	two- or four-days-old	Ramadan et al., (1992); Quimio & Walter (2000)
	Ichneumonidae	*Diadegma semiclausum*	*Mating Occurrence*	< 12 hours	Khatri et al., (2008)
Simple Eusocial	Vespidae	*Polistes lanio*	*Complete Reproductive Maturation*	16 days-old[1]	Gobbi (1975) in Giannotti (2004)
	Apidae	*Bombus terrestris*	*Mating Occurrence*	six- or ten- days-old	Duchateau & Mariën (1995); Tasei et al., (1998)
Advanced Eusocial	Apidae	*Apis mellifera*	*Complete Reproductive Maturation*	12 or 16 days-old	Ruttner (1976); Rhodes (2002)
	Vespidae	*Vespa velutina*	*Complete Reproductive Maturation*	10.3 days-old (mean)	Poidatz et al., (2017)
	Formicidae	*Linepithema humile*	*Mating Occurrence*	Immediate to one-day-old[2]	Passera & Keller (1992)

JH appeared to both regulate dispersal and reproductive maturation, with treatments of methoprene causing earlier departure and accelerated accessory gland fluid area increasement. JH is therefore likely to play a similar role in *P. lanio* males as it does in Apidae males (Giray & Robinson, 1996; de Oliveira Tozetto et al., 1997; Harano et al., 2008; Sasaki et al., 2012; Sasaki & Nagao, 2013). In male insects, JH may play a regulatory role in not only dispersal (e.g. milkweed bugs *Oncopeltus fasciatus* – Caldwell & Rankin, 1972) and courtship behaviour (e.g. *Drosophila melanogaster* – Wijesekera et al., 2016), but also physiological sexual maturation such as in accessory gland activity (e.g. red flour beetles *Tribolium castaneum* & *D. melanogaster* – Wilson et al., 2003; Parthasarathy et al., 2009). Accessory gland fluids facilitate sperm transfer, but in many species also contain a concoction of biochemicals (such as sex peptides) that increase mating success and influence post-copulation female behaviour (Gillott, 2003). However, our current experiment is not able to determine whether dispersal linked to JH (or a JH analogue) occurs due to enhanced sexual maturation (specifically the increase in accessory gland fluid), or due to the effects of the hormone on behaviour independent of reproductive state. Additionally, our tests only recorded binary outcomes of ultimate behaviours, whether a male dispersed or not. How increase levels of JH influence male behavioural profiles on the nest, such as male effect with brood and female nestmates, is unknown. Irrespective of this, it is apparent that JH plays a role in regulating male dispersal in *P. lanio*.

Delayed dispersal presents the possibility that there may be two distinct phases in the life history of males, that of an immature period on the natal nest, and a sexually mature period in dispersed males. Males are regarded as a drain on group resources, as males are not thought to contribute directly to group productivity (Trivers & Hare, 1976). However, delayed dispersal may provide an opportunity for selection on males to enhance their indirect fitness, such as by contributing to nest function (Cameron, 1986; Giannotti, 2004) and therefore increasing inclusive fitness. We investigate the possibility that males may contribute to the nest functionality in Chapter 6.

In conclusion, this chapter demonstrates that natal dispersal of males in *P. lanio* is delayed, is age determined (with some nest variance), that dispersal correlates with reproductive maturation, and that it is regulated by JH. This is the first study (to our knowledge) on factors relating to male dispersal rates in *Polistes* paper wasps. Our findings raise questions on dispersal behaviour of males in social hymenopteran societies in general, and what the potential adaptive (or maladaptive) significance of delayed male dispersal may be.

5.6. Appendix List

Associated appendixes: Appendix VI – male wing length correlates with body size measurements; Appendix VII – male eye colouration with age; Appendix VIII – mite species found on dispersed off-nest male.

Chapter 6. Young adult males in the Neotropical paper wasp *Polistes lanio* contribute to brood care on natal nests

Contributions: R. Southon, A. Radford, & S. Sumner designed the study; R. Southon conducted the fieldwork, with help from S. Sumner and field assistants L. Crowley & S. Morris; R. Southon conducted behavioural observations, dissections, and analyses.

6.1. Abstract

Inclusive fitness may be maximised directly by reproducing, or indirectly by helping raise kin. In eusocial societies, the evolution of behaviours that maximise indirect fitness gains over direct fitness is found in members of the worker caste, which forgo reproduction to help raise siblings. The worker caste in the eusocial Hymenoptera is inherently female-biased, with adult males typically dispersing soon after emergence to mate. However, occurrences of helping-like behaviours such as food distribution, nest guarding and nest thermoregulation before natal dispersal have been reported among hymenopteran males. If (and how) males actively help, has received little attention in the current literature. Here we show that natal adult males in the Neotropical eusocial paper wasp *Polistes lanio* perform an overt helping behaviour, through the distribution of food to nestmate brood. In observing male behaviour, natal males that received food from foraging females or experimentally from an observer had more frequent and longer food provisioning interactions with the brood. However, helping effort was negatively associated with age: older natal males were less likely to accept experimental food-offerings and to distribute food to brood. A decrease in helping effort with age may represent a transition between strategies, with young natal males investing in indirect fitness, and older males who subsequently disperse to mate investing in direct fitness. The mechanisms regulating male helping, however, remain unclear as we found no link between helping effort and variance in reproductive maturity. We show that helping is a trait associated with young males in *P. lanio*; further studies are needed to determine to what extent male helping is altruistic or mutually beneficial, and how widespread this trait is across the Hymenoptera.

6.2. Introduction

A major life history event that individuals of cooperatively breeding societies will experience is the choice (or forced action) of staying or leaving their natal group. If philopatric, individuals may benefit from helping closely related kin (Hamilton, 1964; Maynard Smith, 1964) or the inheritance of resources in the current location (Leadbeater et al., 2011; Field & Leadbeater, 2016). If dispersing, individuals may exploit new resources for their own breeding effort, which may have been suppressed in the previous group (Matthysen, 2012; Hansson & Åkesson, 2014). Evolution will favour the option that provides the highest inclusive fitness payoff. However, fitness payoffs are unlikely to be uniform through an individual's lifespan. For example, if mating opportunities are temporary limited, then alternative sources of fitness may be selected for when optimal fitness via mating is unavailable. Thus, risky irreversible decisions such as natal dispersal to mate may be delayed, if suboptimal fitness alternatives outweigh the benefit of immediate dispersal (Koenig et al., 1992). In observing behaviour at different life stages, sources of fitness that cumulate lifetime inclusive fitness may be mapped, and a greater understanding of a species evolutionary history revealed.

Kin selection is a powerful selection force that can drive individuals to be philopatric, due to gains in indirect fitness via helping related groups members (Hamilton, 1964). Behaviourally, kin selection is expressed as alloparental care, the act of helping another individual's offspring (Wilson, 1975). Though alloparental care need not always involve related individuals, such as reciprocal altruism (Trivers, 1971) or misdirected care (e.g. brood parasitism – Lyon & Eadie, 2000); care towards related individuals may be more widespread as it does not rely on complex social interactions (Trivers, 1971; West-Eberhard, 1975; Riedman, 1982). However, kin-alloparenting in some individuals may only be temporary (Koenig et al., 1992). For example, young grey jays *Perisoreus canadensis* help younger siblings before ultimate dispersal, whilst they themselves are still dependent on the breeding parents (Waite & Strickland, 1997). The eusocial Hymenoptera are characterised by the alloparental behaviour of sisters helping to rear related siblings, and the decision for females in totipotent species to either stay and help or leave to found new nests (Queller & Strassmann, 1998). In contrast to the altruistic feats of their sisters, males in these societies are expected to be void of alloparental behaviour.

The re-direction of ancestral maternal care (Queller, 1994; Bourke & Franks, 1995) to alloparental helping by an effectively sterile female worker caste is thought to be a key mechanism by which a female worker caste evolved in the eusocial Hymenoptera (West-Eberhard, 1987; Linksvayer & Wade, 2005; Toth et al., 2007). The absence of a male worker caste among the eusocial Hymenoptera may be attributed to four interconnecting factors which relate to hymenopteran evolutionary history. First, paternal care is absent at the transition into eusociality (Andersson, 1984; Bourke & Franks, 1995; Queller, 1997; Hughes

et al., 2008). Second, males may lack morphological characteristics that females possess that allow for certain helping acts, such as a stinger for defence or pollen carrying apparatus for foraging (Starr, 1984a; Starr, 1985a). Third, relatedness asymmetries from haplodiploidy sex-determination (haploid males and diploid females) in which males do not have sons, results in higher fitness payoffs for males to mate and produce daughters (life-for-life $r = 1.0$) than to care for offspring or siblings ($r = 0.5$) (Hamilton, 1972; Trivers & Hare, 1976; Bourke & Franks, 1995). By extension to this reasoning, the final factor being that haplodiploidy promotes sib-rearing alleles in the female rather than male sex (Reeve, 1993).

Despite plausible explanations for why helping is female-biased in the social Hymenoptera, natal male helping-like behaviours have been reported in several species of social ants, bees, and wasps (reviewed in Chapter 1, Table 1.1). These behaviours include: food distribution to younger members of the nest, commonly observed in *Polistes* paper wasps, whereby males may take forage from worker females and subsequently provision larvae (Cameron, 1986; Giannotti, 2004); nest defence, with observations of males in the *Microstigmus nigrophthalmus* wasp chasing away nest intruders (Lucas & Field, 2011); and contributions to nest thermoregulation – either indirectly by generating heat through their mere presence in the nest (Harrison, 1987; Kovac et al., 2009), or directly by actively fanning or incubating brood (Cameron, 1985; Cameron, 1986; Giannotti, 2004). Male helping seems most prevalent in natal individuals, and hence is likely directed towards siblings. Only a few solitary wasp species, such as *Trypoxylon politum*, show paternal behaviours. (Hamilton, 1972; Peckham, 1977; Tsuneki, 1970; Brockmann, 1980; Hook & Matthews, 1980; Starr, 1984a; Brockmann & Grafen, 1989). Males of *T. politum* guard a nest containing brood-offspring, and this has likely evolved as a secondary function of mate-guarding behaviour (Brockmann & Grafen, 1989). In natal helping males, as helping is likely directed towards siblings, there is potential for indirect fitness benefits by contributing to the productively of the kin-group (Sen & Gadagkar, 2006; Lucas & Field, 2011). However, male helping is unlikely to be 'truly altruistic'. Male helping behaviours may be mutually beneficial or a by-product, increasing the fitness of both the actor and recipient, such as if males take food for themselves and distribute the remaining to brood or defend themselves and by extension nestmates (Cameron, 1986). Alternatively, male helping may also be 'weakly altruistic', for example in chasing away intruders when the energy expenditure or threat of harm to the male is minimal, posing little to no cost to the adult male and benefitting related brood (Gadagkar, 1993).

Hymenopteran males are typically regarded as a drain on natal group resources, at least from the perspective of female fitness, and hence there is likely to be strong selection for them to leave the nest upon emergence (Trivers & Hare, 1976; Brian, 1983; Starks & Poe, 1997). It is not known, therefore, why males are permitted to stay in some species, but possible explanations depend on which sex ultimately dictates male dispersal. If female nestmates control when males leave the nesting group, then males may 'pay-to-stay' on the nest by

helping. In a pay-to-stay relationship, the benefit to the male is in minimising exposure to extranidal predation or pathogens before reproductive maturity and dispersal, whilst female nestmates benefit from the males' help (Kokko et al., 2002; Bergmüller et al., 2007). Pay-to-stay is demonstrated in cooperatively breeding cichlids *Neolamprologus pulcher*, in which reproductive subordinates help raise the offspring of the dominant individual until vacant breeding sites are available for themselves (Bergmüller et al., 2005). However, pay-to-stay relationships are typically between unrelated individuals, subordinate helpers of *N. pulcher* decrease helping effort with higher relatedness to the reproductive dominant, likely as kinship decreases the cost of lazy helpers (Hamilton & Taborsky, 2005; Zöttl et al., 2013b). Alternatively, if hymenopteran males control when they leave the nest, helping may be a way for males to maximise inclusive fitness by contributing to the rearing of relatives instead of dispersing (Hamilton, 1964), or 'making the best of the current situation' until optimal dispersal conditions with reproductive maturity are present (delaying dispersal).

Male helping may be age-determined. Hölldobler (1966) observed that immature (likely natal) male ants of *Camponotus spp.* and *Formica polyctena* help in nests by exchanging liquid food with adult nestmates, but that male fluid-exchange acts diminish with sexual maturity. As most reports of hymenopteran male helping is in natal individuals, and of relatively young ages – such as brood provisioning by *Polistes* males between zero and 31 days-old (Chapter 1, Table 1.1) – helping males are likely to be sexually immature. Helping by young natal males could therefore be opportunistic. Males in social Hymenoptera often delay dispersal (Chapter 5), and could remain with the natal group until sexual maturity or until there are receptive females available for mating, whereupon the males might disperse from the nest (Hamilton, 1972; Moors et al., 2009). The advantage is that males only leave the nest when they can maximise their mating success; this means they could optimise both their personal direct fitness and the indirect fitness of their relatives. If during their time on the natal nest, males also contribute to the nesting group, then this potentially benefits male and female nestmates by enhancing indirect fitness for all parties. Male fitness strategies may be stratified into two phases: phase 1, a period when males are young and possibly immature, males should invest in indirect fitness by contributing to their natal colony's productivity; phase 2, a period when males are older and likely sexually mature, males should invest in direct fitness by dispersing to mate with unrelated females away from the natal nest. Thus, in hymenopteran species in which helping males are young, helping behaviour by males may be part of a temporal phenotype in a male's life history that is regulated by age and possibly sexual maturation (Chapter 1, Figure 1.2). The idea of male helping directly associated with age has received little empirical testing.

Here we experimentally test whether males help via food distribution to brood in a Neotropical eusocial paper wasp, *Polistes lanio*. We also investigate the factors that influence the expression of affirmed male helping behaviour. We studied brood provisioning behaviour in *P. lanio* for three reasons. First, helping in the form of food distribution to brood has been

observed in males of *P. lanio* previously (see Giannotti, 2004). Second, the physical act of taking food into brood cells likely represents overt helping effort, whereas behaviours such as defence and thermoregulation may be by-products of more selfish actions (Lucas & Field, 2011). Third, males in tropical species are produced throughout the year (Giannotti & Machado, 1994a), and nests of *P. lanio* exhibit high reproductive skew among female and male brood (Chapter 3). Thus, emerging males are likely to be closely related to the brood on their natal nest, offering the possibility to gain indirect fitness by helping. Finally, delayed male natal dispersal was found to be associated with age and reproductive maturation in *P. lanio* (Chapter 5). As male dispersal is delayed, young sexually immature males are present on the natal nest, presenting a temporal window in which males may express helping behaviour. As food distribution behaviour is reliant on the chance to receive forage from a foraging female returning to the nest with food, we experimentally simulated foraging returns by offering males on the nest a standardised food source.

We address three main questions. Question 1: do males help on the nest under natural conditions (i.e. if they accept food from unmanipulated, foraging females), and do males help when experimentally offered food (i.e. from an experimenter)? We define helping behaviour in terms of food distribution to brood: this would suggest that males actively contribute to brood care, and thus invest in indirect fitness. Question 2: does male helping effort decrease with age? We experimentally offered a food source to individual males each day they spent on the natal nest: a change in helping effort (both willingness to accept food and subsequent distribution) with age might suggest that male fitness investments are temporally linked. Question 3: is helping effort associated with sexual maturity? Since males were found to be sexually immature on the natal nest (Chapter 5), variance in helping effort may be associated with reproductive development.

6.3. Methods

6.3.1. Study sites and monitoring methods

Experiments were conducted with *P. lanio* males on their natal nests during the wet season July–September 2015, Trinidad, Trinidad & Tobago. Eleven nests were used, located in three semi-rural areas utilised throughout this thesis (of approx. two hectares each): four nests at Verdant Vale (site VV), Blanchisseuse Rd, 2015 (10°41'5.44"N, 61°17'24.95"W); three nests at Eastern Main Rd (site EM), 2015 (10°39'1.21"N, 61°15'9.63"W); and four nests at Cumuto Tamana Rd (site CT), 2015 (10°34'48.01"N, 61°14'38.06"W) (Table 6.1). Nests were found along the eaves of houses, in abandoned buildings, and in livestock pens. Post-emergence nests (established nests with emerged adult offspring) producing males, and that had larval brood present, were chosen for study.

Upon discovering a nest, we made a brood map of the nest. Brood maps were used to record the positions of brood within the nest and their developmental stage. Each cell was assigned a category of egg, larva, pupa, empty, or parasitised (containing a visible signs of nest parasitism, e.g. by Ichneumonoidea or Lepidoptera).

All males received an individual identity mark at nest discovery, with newly emerged adult males marked throughout the monitoring and experimental period. Marking involved removing males from the nest with forceps, and applying a four-colour spot combination to the dorsal thorax using extra-fine-tip Uni POSCA markers. After marking, wasps were returned to the nest with forceps. Male wasps marked at the start of monitoring were excluded from further study, as their ages were unknown. A selection of focal males that emerged on nests during the monitoring period were included in the experiment. Male wing length was measured at the time of marking. Wing length is a general indictor of body size (in females, West-Eberhard, 1969; in males, see Appendix VI). Wing length was measured using digital calipers (millimetres to 2 d.p.), as a straight line between the intersection of a tegula and wing to the furthest apex tip. This allowed male body size to be included in subsequent models (size does not change once individuals emerge from pupation), as size may predisposition individuals to certain behavioural tasks (Hölldobler & Wilson, 1990). When wings were creased, they were unfolded before measurement. Mean ± SE male wing length in the population was 18.89 ± 0.15 mm (Question 1 and 2 dataset) and 17.86 ± 0.11 mm (Question 3 dataset).

In order to identify newly emerged males, nests were checked at the beginning of each day for unmarked males and hatched pupal cells. Newly emerged adult males were identified from their shiny black eyes colouration (Appendix VII), lack of wing wear (Garcia & Noll, 2013), and appearance that coincided with a hatched pupal cap. After marking, nests were left for at least 30 min before any experimental trials were conducted, to allow wasps to settle. Using this protocol, experimental trials used only focal males that were both natal to the nest and had known ages.

6.3.2. Experimental setup

Sixty-five males across 10 nests (Table 6.1) were used to test Questions 1 and 2: *natural trials* were used to test whether helping behaviour naturally occurred in the population (Question 1); *food-offering trials* were used to test whether males helped when experimentally offered food (Question 1), and if helping effort decreased with age (Question 2). We offered food to males to test whether helping changed over time, as preliminary observations detected that helping was associated with female foraging trips (which were rare). Each group of focal males on a nest received both one *natural* and one *food-offering trial* per day between the hours of 08:00 and 18:00, with trials filmed for 25 min after manipulation so that the behaviour of

males could be analysed. The order of trial was first assigned randomly (by coin flip), with the group receiving a *natural* or *food-offering trial* together, and thereafter trials sequentially performed on subsequent days until all focal males had dispersed (departure age ranged from one- to five-days-old). The second trial each day for a group commenced 10 min after the filming of the first trial finished. A maximum of 10 focal males per nest were used at any given time on a nest. This reduced how much food the nest received during and between *food-offering trials*.

Seventy-two individual males across seven nests (Table 6.1) were used to test whether helping effort was associated with sexual maturity (Question 3). In this separate experiment, a *food-offering and collection trial* was conducted – the behavioural response of males was analysed, and reproductive state assessed from dissections.

6.3.3. Question 1: Do Males Help? (*natural trials*)

Observations of how males responded to female foraging trips and if helping normally occurred were analysed (for example, if an artefact of offering food in *food-offering trials*). A *natural trial* consisted of holding empty forceps approx. 5 cm away from the front (defined as where the brood were visible) and then back of the nest for 1 min each side, or vice versa. Order of forceps introduction was initially randomised by a coin flip, and then sequentially ordered for subsequent days. After 2 min total of holding forceps to the nest, forceps were taken away and the nest was filmed for 25 min in order to record interactions of males with cells containing brood (6.3.5. Filming and Behaviour Scoring). We held forceps to the nest as a control, to determine that the methods use in the food-offering experiments were not in themselves eliciting behavioural artefacts.

6.3.4. Question 1 and 2: Do males help, and is helping influenced by age? (*food-offering trials*)

Food-offering trials allowed the chance for focal males to accept or reject experimentally offered food, observe if they fed accepted food to brood (Question 1), and test whether this potential helping effort decreased with age (Question 2). Food consisted of *P. lanio* larvae, skinned and gut removed before rolling into a 1.5 mm diameter ball. Brood cannibalism is known in this species, and therefore is a natural dietary source (Giannotti & Machado, 1994a). Larvae used for food were obtained from nests at other sites, at least 5.6 km away, and hence unlikely to be closely related to trial nests. Before food was offered to males, the nest was watched for at least 5 min to check that males weren't already engaged in food-distribution behaviour. For each *food-offering trial*, a focal male was selected at random, and a food ball held in forceps directly in front of its mandibles for 1 min. If the male moved around the nest, then movement was matched by the experimenter to keep the food ball in front of the focal male's mandibles. Food was scored as 'accepted' if the focal male grabbed the food ball in its

mandibles – if this happened the male was allowed to take the food from the forceps. The time to accept was recorded as from the moment the food was first introduced until (if) the focal male grabbed the food ball. Food was scored as 'rejected' if the focal male ignored the offering for the entire 1 min food-offering period. When one male had been offered food, the next focal male was randomly selected for presentation and so forth, until all focal males for that day received an offering. As the number of focal males included in trials on a single nest was limited to 10 males at any given time (6.3.2. Experimental setup), up to 10 offerings could be made on the nest per trial between 0 and 10 min. Nests were filmed for the entire period, so that the behaviour of each male could be analysed for 25 min after offerings.

6.3.5. Filming and scoring of behaviour

Natural and *food-offering trials* were filmed using SONY® HDR-CX220 and Panasonic® HC-V250EB-K camcorders. Nests were filmed for 25 min from either the end of forceps introduction (*natural trials*) or the end of the first food-offering outcome (*food-offering trials*). The front of the nest was filmed, as this is where feeding interactions with the brood happen. Subsequent behaviour of males was scored using the ethogram presented in Table 6.2, focusing on food-distribution behaviour. Direct contact between brood and adult wasps could not be observed, so interactions of wasps with a cell was used as a proxy for food distribution to brood (referred to as 'male–cell interaction'). Because trials were conducted on groups of males at the same time, and in *food-offering trials* one focal male could receive food before the other, we staggered 25 min observations so each focal male received equal time in scoring behaviours. We report descriptive values for what category brood males interacted with, and its relation to the overall brood composition of the nest. Behavioural scoring was conducted blind to focal male ages.

6.3.6. Question 3: Is helping linked to sexual maturity?

Food-offering trials were used to assess whether helping effort was associated with sexual maturity, independently of age. As per experiments for Question 1 and 2, newly emerged natal males were marked daily (for at least four days). On the final day for a given male, a *food-offering trial* was conducted. The outcome of the offering (either accept or reject) was recorded, and food-distribution behaviours watched for 30 min by an observer, recording the first behaviour related to distribution (ethogram in Table 6.2). After the 30 min observation period, focal males were collected from the nest and stored in 70% ethanol (and subsequently stored long-term at -20°C until dissections of the reproductive organs were made). Dissections were carried out as per methods in Chapter 5. Male dissections were conducted in phosphate-buffered saline, using a Leica M165 C stereo microscope with a Leica IC80 HD digital camera attachment. Dissections were performed by cutting a conducted in phosphate-buffered saline, using a Leica M165 C stereo microscope with a Leica IC80 HD digital camera attachment. Dissections were performed by cutting a rectangular section in the ventral side of the

abdomen and peeling back tergites, removing the testes, and the pair of seminal vesicles with accessory glands. Removed organs were prepared on a slide, and photographs taken. Using ImageJ 1.51j8, two-dimension measurements were taken of the area around the outer layer of the testes (mm^2 to 2 d.p.), and the area of the fluid inside the seminal vesicles and accessory glands separately (mm^2 to 2 d.p.) (Chapter 5, Figure 5.1). Fluid area of the seminal vesicles and accessory glands is reported as the mean area between paired organs respectively, as the simultaneous function of ejaculation is not known. In analysis, fluid area is referred in relation to mSV (mean seminal vesicle) and mAG (mean accessory gland). Scoring and measurements were conduct blind to the age of the individual.

Table 6.2. Ethogram of male behaviours that were relevant to food-provisioning on the nest. Larvae are classified as: Small = larva has no contact with hexagon cell walls beyond attachment point; Medium = larva partially fills cell, in contact with parts of hexagon cell wall; Large = larva filling cell, touches all parts of hexagon cell wall.

Behaviour	Type	Who/What	Description
Male Interactions with Cells (male-cell interaction)	Count and Duration	Small/Medium/Large Larvae	An individual places its head (fully/partially) into a cell for longer than 1 second. Count and duration ends once head is fully removed. A repeated cell interaction on the same cell is recorded as a new event if the head is fully removed between interactions.
Adult Solid Food Exchange	State	Adult Nestmates: Female or Male	*Gain* : Focal male takes either patially or fully takes a solid food item from another wasps mouthparts. There may be physical contact with the recipiant wasp, or only contact with the food. Count event ends once focal male has no contact with the food held by the other wasp for longer than 5 seconds. Repeated interaction can occur on the same food item.
			Loss : as above, with another wasp taking food from the focal wasp. May include natural forage or offered food.
			Gain-Loss : combination of *Gain* and *Loss* , focal male gains non-offered food, but subsequently loses it before cell interactions.

Table 6.1. Characteristics of sampled post-emergence nests. Sites: VV = Verdant Vale; EM = Eastern Main Rd; CT = Cumuto Tamana Rd. Number of larvae: Small/Medium/Large. Number of adults across trials, mean count of female and male adults on the nest between daily censuses.

		Number of Focal Males					Nest Comb and Group Size During Trials								
Nest ID	Site	Number of Focal Males (Hyp. 1–2)	Median Age of Focal Male Dispersal (Hyp. 1–2)	Dates when nest in use (Hyp. 1–2)	Number of Focal Males (Hyp. 3)	Dates when nest in use (Hyp. 3)	Date of Brood Map	Total Number of Cells	Total of Eggs	Number of Larvae S/M/L	Number of Pupae	Number of Empty Cells	Number Parasitised Cells	Mean Number of Adult ♀ (Hyp. 1–3)	Mean Number of Adult ♂ (Hyp. 1–2)
#01	VV	9	four-days-old (IQR = 3.0)	23 Jul – 01 Aug	-	-	23 Jul	126	23	0/5/12	18	68	0	9.3 ± 0.9	3.3 ± 0.7
#02	VV	14	six-days-old (IQR =1.0)	23 – 30 Jul	-	-	23 Jul	172	23	0/0/8	29	112	0	7.7 ± 0.8	9.7 ± 1.2
#03	CT	5	three-days-old (IQR = 1.0)	24 – 29 Jul	11	30 Aug – 08 Sep	30 Aug	377	31	30/74/46	92	132	2	29 ± 1.2	4.5 ± 1.2
#04	CT	10	three-days-old (IQR = 1.5)	09 – 15 Jul	11	20 Aug – 08 Sep	09 Jul	427	71	33/79/38	57	149	0	13.9 ± 0.7	8.8 ± 1.3
#05	CT	8	three-days-old (IQR = 0.3)	22 – 25 Aug	38	01 – 07 Sep	23 Aug	491	132	27/66/71	130	65	0	30.0 ± 2.0	9.8 ± 1.8
#06	CT	5	two-days-old (IQR = 0.5)	27 Jul – 01 Aug	2	08 – 10 Sep	27 Jul	107	33	19/22/20	13	0	0	6.8 ± 1.0	1.3 ± 0.5
#07	EM	2	two-days-old (IQR = 1.0)	23 – 26 Aug	-	-	23 Aug	208	36	12/20/26	44	70	0	12.3 ± 1.1	1.3 ± 0.3
#08	EM	5	four-days-old (IQR = 1.0)	23 – 28 Aug	-	-	23 Aug	306	0	0/0/5	56	245	0	20.8 ± 3.2	10.0 ± 1.3
#09	EM	-	-	-	5	04 – 06 Sep	-	-	-	1/3/0	-	-	0	-	-
#10	VV	6	four and half-days-old (IQR = 1.8)	03 – 09 Sep	4	19 – 23 Sep	19 Sep	181	58	7/3/18	55	40		20.7 ± 1.7	3.7 ± 0.6
#11	VV	1	one-day-old(IQR = n/a)	06 Sep	1	06 – 10 Sep	06 Sep	117	18	16/10/14	8	51	0	13.5 ± 0.5	1.0 ± n/a
							Mean	251.2	42.5	13.2/25.6/23.5	50.2	93.2	0.2	16.5	5.8
							± SE	44.1	11.8	3.8/9.5/6.3	12.0	21.9	0.2	2.6	1.2

6.3.6. (Statistical Analysis) Question 1: Do males help?

The following models used data from *natural trials* and *food-offering trials*.

First, we simplified the fixed effect variable of 'food state' for subsequent models. Food state consisted of two main factors combined, with 10 levels in total (Factor 1 trial type – three levels: whether it was a *natural trial* with no pre-trial food or *food-offering trial*, the latter of which a male could either accept or reject an experimental food-offering; Factor 2 solid food exchange, see Table 6.2 – four levels: whether during the 25 min observational period a male gained additional food from a foraging female, and if a male lost food to another adult wasp, either which it had accepted from experimental offerings or gained from foraging female female). We ran a zero-inflated generalized linear mixed model (poisson distribution) (GZLMM) for count data as the response (i.e. how many interactions a focal male had with cells containing larvae within the 25 min period). The fixed effect was food state. The model accounted for multiple repeated measures on the same individuals, by fitting trial number by wasp ID as a random effect term.

To assess the occurrence of male helping within the context of either gaining food from foraging females in *natural trials* or accepting experimental food during *food-offering trials*, we first analysed male–cell interaction as counts, by repeating the a zero-inflated GZLMM model above with the simplified fixed effect of food state (four levels: *natural trial* – no food gained foraging females; *natural trial* – food gained from foraging females (combining food gained, and lost to another adult wasp); *food-offering trial* – accepted experimental food-offering (combing whether additional food was gained from a foraging female and/or if any food was lost to another adult wasp); *food-offering trial* – rejected experimental food-offering). Second, to assess male–cell interactions as a duration, we ran another GZLMM (binominal distribution) with the response variable of how long (seconds) a focal male interacted with cells containing larvae within the 25 min period (transformed as a proportion). The same independent variables as for count data were used for fixed and random effects.

We tested separately whether male–cell interaction counts and durations (transformed) of males that gained food from foraging females in *natural trials* or accepted food from experimental offerings in *food-offering trials* (including subsequent loss to adult wasps) correlated with wing length using a Spearman's rank (Spearman) tests.

6.3.7. (Statistical Analysis) Question 2: Is helping influenced by age?

The following models used data from *food-offering trials*.

First, we analysed acceptance and rejection behaviour, and whether willingness to accept food changed with age. We ran a GZLMM (binominal distribution), with the binary response variable of a focal male accepting or rejecting the food within the 1 min food-offering period. The fixed effects for the model were male age (day) and wing length (mm), with the random effect of natal nest origin (intercept). The model accounted for multiple repeated measures on the same individuals, by fitting trial number by wasp ID as an additional random effect term.

Second, using the subset of males that accepted the food offering, a general linear model (GLMM) was run to assess whether latency to accept food offerings changed with age. Latency to accept was log10(duration + 1) transformed. The same independent variables (as for binary GZLMM data above) were used for fixed and random effects.

Third, we analysed male–cell interactions in *food-offering trials* in relation to age. We ran a zero-inflated GZLMM (poisson distribution), with the response variable of how many interactions a focal male had with cells containing larvae within the 25 min period after food was offered. The fixed effects for the model were the response to the food offering (whether it accepted or rejected the offering), male age (day), and wing length (mm). The model accounted for multiple repeated measures on the same individuals, by fitting trial number by wasp ID as a random effect term.

Finally, to assess male-cell interactions as a duration during *food-offering trials*, we ran a GZLMM (binomial distribution) with the response variable of how long (total) a focal male interacted with cells containing larvae within the 25 min period (as a proportion). The same variables as for count data above, were used for the fixed and random effects.

6.3.8. (Statistical Analysis) Question 3: Is helping linked to sexual maturity?

The following models used data from *food-offering* and *collection trials*. To investigate whether food acceptance and subsequent male–cell interactions were associated with reproductive organ size, multiple GZLMs (binominal distribution, logit model) were performed. A set of three models tested whether the response variable of a male accepting or rejecting food was associated with fixed effects of testes size, mSV, and mAG fluid area separately (as reproductive organ sizes are not independent of one another). Another set of three models tested whether the response variable of a male keeping food (including losing the food to another wasp) or placing it in a cell with a brood (male–cell interaction) was associated with

fixed effects of testes size, mSV, and mAG fluid area separately. All models additionally accounted for the fixed effect of wing length (mm).

6.3.9. General Protocols of Statistical Analysis

We used histograms and Q–Q plots to assess for deviations from a normal distribution where applicable. Zero-inflated GZLMMs were used, which treats excessive zeros as a separate subpopulation – as preliminary GZLMM analysis indicated overdispersal of count data. Parametric model fit was assessed by checking a residual vs fitted value plot (GLMMs). The *p* values for overall fixed effects were calculated using a likelihood ratio test of the model with and without the target factor. Post-hoc pairwise tests for multilevel fixed effects used chi-squared test with Holm–Bonferroni adjusted *p* values for count data zero-inflated GZLMMs (poisson distribution), and least square means (LS means) for duration data GZLMMs (binominal distribution) by back-transforming and scaling the response variable (Tukey-method adjusted *p* value comparisons). Variance of the random effects on the models are reported (with standard deviation, SD).

Analyses were performed with R 3.3.3 (R Core Team, 2017), using packages 'lme4' (Bates et al., 2015), 'RLRsim' (Scheipl et al., 2008), 'phia' (De Rosario-Martinez et al., 2015), 'lmerTest' (Kuznetsova et al., 2016), 'multcomp' (Hothorn et al., 2008), 'Hmisc' (Harrell Jr et al., 2017), 'car' (Fox & Weisberg, 2011), 'glmmADMB' (Fournier et al., 2012; Skaug et al., 2013), 'lsmeans' (Lenth, 2016). All analyses were tested at a significance of $\alpha = 0.05$, with averages reported as median with IQR or mean ± SE when applicable.

An alternative zero-inflated GZLMM model is detailed in Appendix IX, which provides parameter estimates of both parts of the zero-inflation.

6.4. Results

6.4.1. Question 1: Do Males Help?

Natural trials: From the observational data, we found evidence that males help naturally by feeding brood on their natal nests when they receive food from foraging females. In *natural trials*, a total of 10 events (in 5% of individual male *natural trials*) of males receiving forage from females were recorded – males never received food from other males. In three of these cases, some of the food was transferred from the male to another female before it proceeded to perform male–cell interactions. In three nests, larvae at different developmental stages were present: on these nests males interacted with large larvae more than any other developmental stage (large 64%; medium 31%; and small 5%). Given these three nests with more than one category of larvae had a mean percentage larval composition of 30% large, 46% medium and 24% small, male feeding appears likely biased towards targeting large larvae.

Food-offering trials: Males that accepted food during the experimental food-offerings also subsequently helped by performing food-distribution behaviour. Out of 193 *food-offering trials*, male accepted food 67% of the time, and subsequently fed brood 59% after (if accepting food). Across seven nests with larvae at different developmental stages present, males interacted with large larvae more than any other developmental stage (large 58%; medium 37%; small 3%; undetermined size = 2%). Given these seven nests with more than one category of larvae had a mean percentage larval composition of 37% large, 43% medium, and 24% small, male feeding with accepted food appears likely biased towards targeting large larvae. There were no recorded events of accepted food being lost to other adult male wasps, only female.

Simplifying the model: First, we simplified the subsequent model by combining levels in the fixed effect variable 'food state' into four levels: *natural trial* – no food gained foraging females; *natural trial* – food gained from foraging females (combining food gained, and lost to another adult wasp); *food-offering trial* – accepted experimental food-offering (combing whether additional food was gained from a foraging female and/or if any food was lost to another adult wasp); *food-offering trial* – rejected experimental food-offering. The food state of the focal male had a significant effect on the number of male–cell interactions it performed (GZLMM: $\chi^2_2 = 99.19$, $p < 0.001$). Individual ID had a variance on the model of 0.47 (SD = 0.69). We identified seven different combinations of food state (out of a possible ten), and based combing levels on lack of significance in Chi post-hoc analysis (see Table 6.3).

Modelling helping behaviour: Males had more male–cell interactions if they had food than if they did not have food (GZLMM: $\chi^2_3 = 130.73$, $p < 0.001$, Figure 6.1a). Males that gained food from returning foraging females in *natural trials* had significantly more male–cell interactions than males that gained no food in *natural trials* (Chi: $\chi^2_1 = 29.01$, $p < 0.001$) and males that rejected offerings in *food-offering trials* ($\chi^2_1 = 16.02$, $p < 0.001$). Males that accepted experimental food offerings in *food-offering trials* also had significantly more male–cell interactions than males that rejected the offering in *food-offering trials* ($\chi^2_1 = 17.82$, $p < 0.001$) and males that gained no food in *natural trials* ($\chi^2_1 = 92.60$, $p < 0.001$). Of males with food, there was no difference in male helping behaviour expressed in the *natural trials* and *food-offering trials* ($\chi^2_1 = 1.42$, $p = 0.234$). However, males that did not gain any food in *natural trials* had significantly more male–cell interactions than those that rejected experimental food offerings in *food-offering trials* ($\chi^2_1 = 5.29$, $p = 0.043$). Males that gained no food in *natural trials* and those that rejected experimental food offerings in *food-offering trials* both had median counts of zero male–cell interactions (IQR = 0.0). There was only one event of a male who rejected food in the *food-offering trials* performing a single male–cell interaction, compared to 16 events in males who gained no forage in *natural trials* (1–3 counts = 15 events, one male trial of 8 counts). The reason for this discrepancy is unknown, though it does suggest that males will sometimes perform some low amounts of interactions with larvae absent of (at least

conspicuous) food. Random effect variance of trial and individual ID on the model was 1.23 (SD = < 0.00).

Table 6.3a. Median counts of male-cell interactions within a trial dependent on the state of receiving food, either from trial condition or from a returning foraging female.

Trial Type	Identified States: Additional Female Food Gained?	Median (IQR) Number of Counts	Percentage of Occurance with Trial Type
natural trial	no food gained	0 (IQR = 0.0)	95% (*n* = 181)
	food gained from female	3 (IQR = 2.0)	4% (*n* = 7)
	food gained from female, and lost	2 (IQR = 0.5)	1% (*n* = 3)
food-offering trial (Accepted Offering)	no additional food gained	1 (IQR = 2.0)	55% (*n* = 96)
	no additional food gained, offered food lost	1 (IQR = 2.0)	10% (*n* = 18)
	additional food gained from female	2 (IQR = 2.0)	3% (*n* = 5)
food-offering trial (Rejected Offering)	no additional food gained	0 (IQR = 0.0)	32% (*n* = 57)

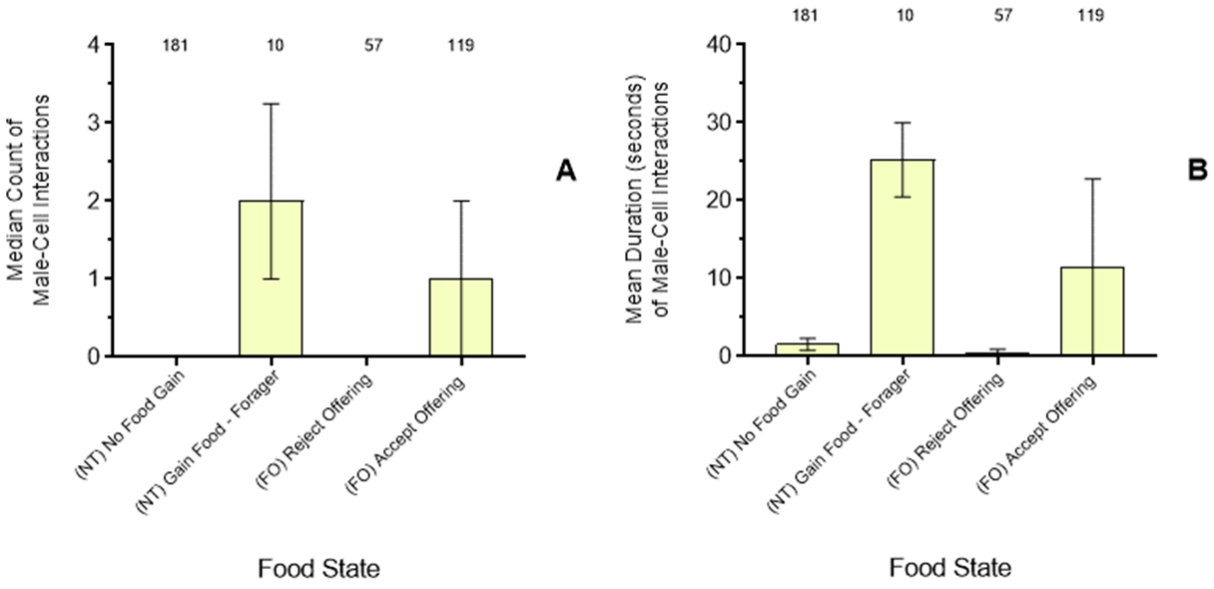


Figure 6.1. Significantly ($p < 0.05$) more male-cell interactions when males have food compared to not in *natural* and *food-offering* trials. Median count with IQR (A) and mean duration ± SE (B) in seconds of male- cell interactions with brood in natural (NT) and food-offering trials (FO). Numbers apex to columns indicate number of trials interaction occurred, independent of number of actual foraging returns.

Table 6.3b. Chi-square post hoc analyses between trial and food states.

Pairing A		Pairing B		Chi-squared Test	Higher Count, Pair A or B
Factor 1a - Trial	Factor 2a - Additional Female Food	Factor 1b - Trial	Factor 2a - Additional Female Food		
natural trial	no food gained	*natural trial*	food gained from female	$\chi^2_1 = 32.78$, $p < 0.001$**	B
			food gained from female, and lost	$\chi^2_1 = 6.38$, $p = 0.138$	n/a
		food-offering trial (Accepted Offering)	no additional food gained	$\chi^2_1 = 15.65$, $p = 0.001$*	B
			no additional food gained, offered food lost	$\chi^2_1 = 9.54$, $p = 0.026$*	B
			additional food gained from female	$\chi^2_1 = 35.75$, $p < 0.001$**	B
		food-offering trial (Rejected Offering)	no additional food gained	$\chi^2_1 = 4.94$, $p = 0.289$	n/a
	food gained from female	*natural trial*	food gained from female, and lost	$\chi^2_1 = 4.34$, $p = 0.373$	n/a
		food-offering trial (Accepted Offering)	no additional food gained	$\chi^2_1 = 3.68$, $p = 0.493$	n/a
			no additional food gained, offered food lost	$\chi^2_1 = 0.40$, $p = 1.000$	n/a
			additional food gained from female	$\chi^2_1 = 0.31$, $p = 1.000$	n/a
		food-offering trial (Rejected Offering)	no additional food gained	$\chi^2_1 = 38.88$, $p < 0.001$**	A
	food gained from female, and lost	*food-offering trial* (Accepted Offering)	no additional food gained	$\chi^2_1 = 0.22$, $p = 1.000$	n/a
			no additional food gained, offered food lost	$\chi^2_1 = 0.37$, $p = 1.000$	n/a
			additional food gained from female	$\chi^2_1 = 1.93$, $p = 1.000$	n/a
		food-offering trial (Rejected Offering)	no additional food gained	$\chi^2_1 = 15.51$, $p < 0.001$**	A
food-offering trial (Accepted Offering)	no additional food gained	*food-offering trial* (Accepted Offering)	no additional food gained, offered food lost	$\chi^2_1 = 0.18$, $p = 1.000$	n/a
			additional food gained from female	$\chi^2_1 = 2.25$, $p = 1.000$	n/a
		food-offering trial (Rejected Offering)	no additional food gained	$\chi^2_1 = 18.70$, $p < 0.001$**	A
	no additional food gained, offered food lost	*food-offering trial* (Accepted Offering)	additional food gained from female	$\chi^2_1 = 0.37$, $p = 1.000$	n/a
		food-offering trial (Rejected Offering)	no additional food gained	$\chi^2_1 = 10.45$, $p = 0.017$*	A
	additional food gained from female	*food-offering trial* (Rejected Offering)	no additional food gained	$\chi^2_1 = 19.53$, $p < 0.001$**	A

Males spent more time interacting with a cell when they were in possession of food (GZLMM: $\chi^2_3 = 975.23$, $p < 0.001$, Figure 6.1b). Males that gained food from returning foraging females in *natural trials* had significantly longer male–cell interactions than males that gained no food in *natural trials* (LS means: Z = 24.29, $p < 0.001$) and males that rejected offerings in *food-offering trials* (Z = 17.02, $p < 0.001$). Males that accepted experimental food offerings in *food-offering trials* also had significantly longer male–cell interactions than males that rejected the offering in *food-offering trials* (Z = 15.74, $p < 0.001$) and males that gained no food in *natural trials* (Z = 29.56, $p < 0.001$). Males that gained food from returning foraging females in *natural trials* had significantly longer male–cell interactions than males that accepted food offerings in *food-offering trials* (Z = -7.13, $p < 0.001$). Males that did not gain any food from returning foraging females in *natural trials* had significantly longer male–cell interactions than males that rejected food offerings in *food-offering trials* (Z = 10.01, $p < 0.001$). Random effect variance of trial and individual ID on the model was 8.17 (SD = 3.40).

In *natural trials*, wing length had no significant relationship with the number or duration of male–cell interactions if a male gained food from a foraging female (Count: Spearman $\rho = 0.19$, df = 6, $p = 0.659$; Duration: $\rho = 0.04$, df = 6, $p = 0.933$). In *food-offering trials*, wing length also had no significant relationship with the number or duration of male-cell interactions if a male accepted food from an experimental offering (Count: $\rho = -0.12$, df = 112, $p = 0.202$; Duration: $\rho = -0.13$, df = 112, $p = 0.161$).

6.4.2. Question 2: Is helping influenced by age?

We found evidence that males were less likely to accept food and feed brood as they aged in *food-offering trials*. Initial acceptance of food in *food-offering trials* was negatively associated with age (GZLMM: $\chi^2_1 = 31.30$, $p < 0.001$, Figure 6.2). There was no significant effect of wing length on likelihood that the food was accepted ($\chi^2_1 = 3.02$, $p = 0.082$). Random effect variance on the model was: natal nest origin = 0.60 (SD = 0.77); individual ID = 1.24 (SD = 1.11).

However, of the males that did accept the food, there was no significant difference in the time taken to accept food (GZLMM: $\chi^2_1 = 0.87$, $p = 0.350$; mean = 21 ± 2 s, Figure 6.3). There was no significant effect of wing length on the latency to accept ($\chi^2_1 = 0.15$, $p = 0.695$). Random effect variance on the model was: natal nest origin = 0.07 (SD = 0.27); and individual ID = 0.05 (SD = 0.23).

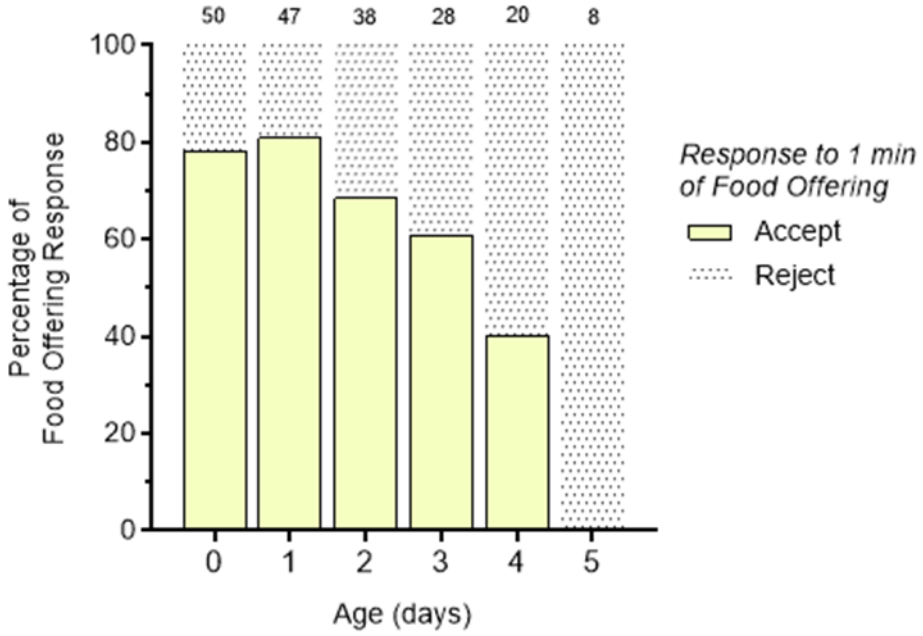


Figure 6.2. Significantly ($p < 0.05$) less food acceptance in *food-offering trials* with male age. Percentage of acceptance of food offerings by age during food offering trials. Numbers apex indicate number of known responses.

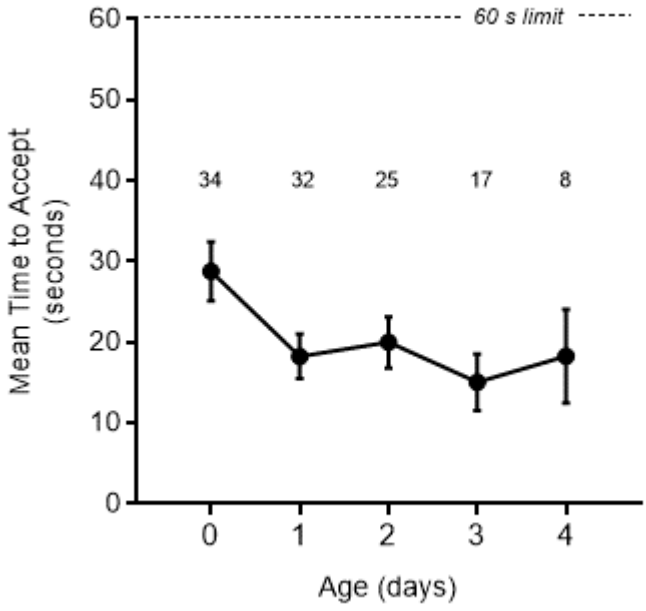


Figure 6.3. No significant ($p < 0.05$) difference in acceptance time over a continuous age range. Mean ± SE time (seconds) to accept food offerings by age during food offering trials. Numbers apex indicate number of known trial-responses.

Age had a significant effect on male–cell interaction counts, with older males engaging less frequently in cell interactions (GZLMM: $\chi^2_1 = 1.05$, $p < 0.001$, Figure 6.4a). Males that accepted food in *food-offering* trials, had fewer male–cell interactions as they aged (Z-value = -3.55, $p < 0.001$). For trials in which males rejected food, there was no significant effect of age on male–cell interaction counts (Z-value = -0.02, $p = 0.980$). Random effect variance of trial and individual ID on the model was 1.23 (SD = < 0.00).

Additionally, males had significantly shorter male–cell interactions with brood as they aged (GZLMM: $\chi^2_1 = 86.16$, $p < 0.001$, Figure 6.4b). Males that accepted food in *food-offering* trials, had shorter total durations of male–cell interactions as they aged (Z-value = -6.538, $p < 0.001$). For trials in which males rejected food, there was no significant effect of age on male–cell interaction durations (Z-value = 0.44, $p = 0.661$).

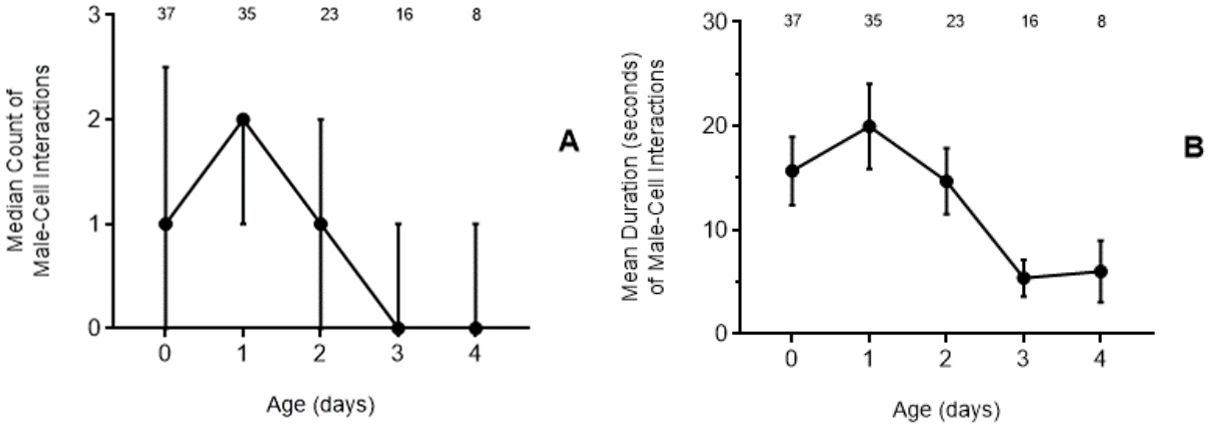


Figure 6.4. Significantly ($p < 0.05$) less male-cell interactions with age in males that accepted food in *food-offering trials*. Median count with IQR (A) and mean duration ± SE (B) in seconds of male-cell interactions with brood when a male accepted the food offering by age in food-offering trials. Numbers apex indicate number of trial-responses.

6.4.3. Question 3: Is Helping linked to sexual maturity?

We found no evidence that helping behaviour was linked to variance in sexual maturation. Measures of reproductive maturity had no significant association with whether males accepted or rejected the food offering in *food-offering and collection trials* (GZLM: testes, χ^2_1 = 0.01, p = 0.917; mSV, χ^2_1 = 1.03, p = 0.311; mAG, χ^2_1 = 0.78, p = 0.377, Figure 6.5). In focal males that rejected the food offering, 98% performed no male–cell interactions. Wing length also had no significant effect on acceptance outcome (testes, χ^2_1 = 0.95, p = 0.330; mSV, χ^2_1 = 1.37, p = 0.242; mAG, χ^2_1 = 1.44, p = 0.229).

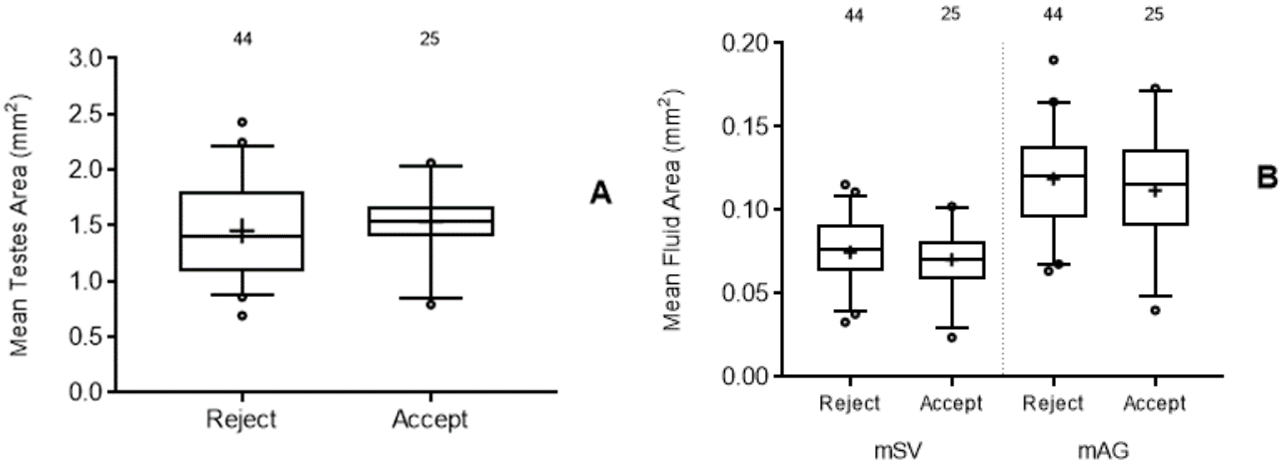


Figure 6.5. No significant (p < 0.05) difference in signs of reproductive maturity in males that accepted or rejected food offerings in *food-offering and collection trials*. Box (25th to 75th) and whisker (5th to 95th) plots with mean centre points of (A) testes area and (B) mSV & mAG fluid area in mm2 between focal natal males that accepted or rejected the food offering irrespective of age during food-offering and removal trials. Points on graph note outliers. Numbers apex indicate number of dissected reproductive organ parts.

Measures of reproductive maturity also had no significant association with whether males kept (including losing the food to another adult wasp) or performed male–cell interactions in *food-offering and collection trials* when they accepted food offerings (GZLM: testes, χ^2_1 = 0.36, p = 0.551; mSV, χ^2_1 = 0.05, p = 0.820; mAG, χ^2_1 = 0.09, p = 0.767, Figure 6.6). Wing length also had no significant effect (testes, χ^2_1 = 0.28, p = 0.595; mSV, χ^2_1 = 0.26, p = 0.612; mAG, χ^2_1 = 0.45, p = 0.503).

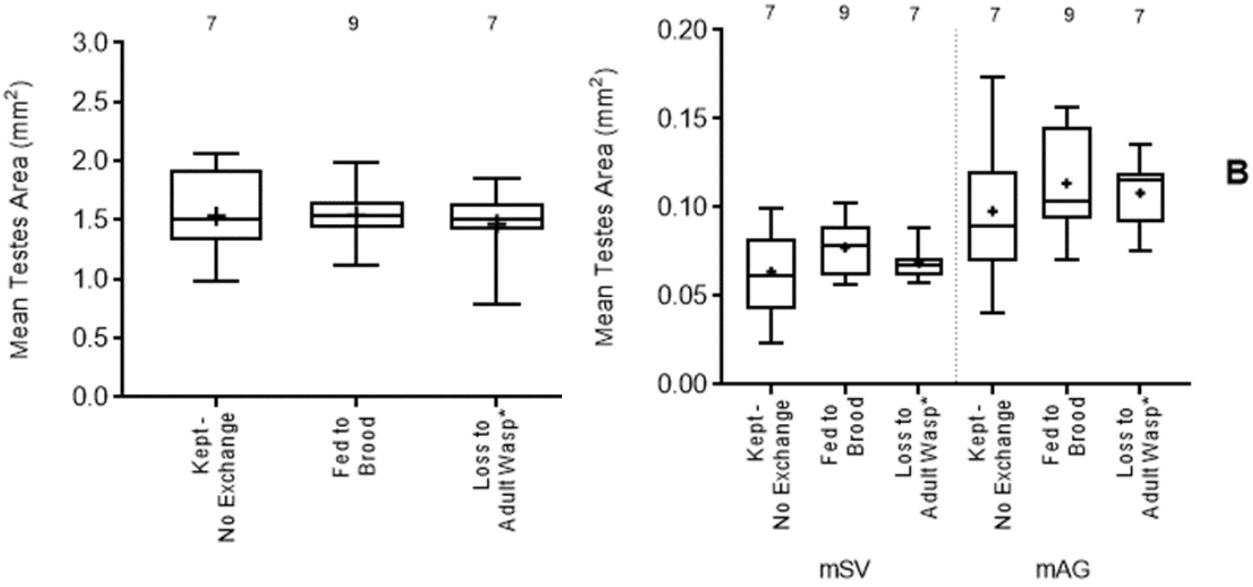


Figure 6.6. No significant ($p < 0.05$) difference in male-cell interactions and other behaviours in those males that accepted food offerings in *food-offering and collection trials*. Box (25th to 75th) and whisker (5th to 95th) plots with mean centre points of (A) testes area and (B) mSV & mAG fluid area in mm2 between focal natal males behavioural response after accepting food offering irrespective of age during food-offering and removal trials. Numbers apex indicate number of dissected reproductive organ parts. * Loss to Adult Wasp state had no brood interactions.

6.5. Discussion

In the social Hymenoptera, helping behaviours that increase indirect fitness are expected to be absent in the male sex. However, in the paper wasp *P. lanio*, we found evidence that males help on their natal nest by provisioning brood. Males that received food from foraging females, or from experimental offerings of food, had more and longer interactions with brood than those males which did not receive food forage or rejected the experimental food offerings. By experimentally offering food to males daily until they dispersed from the nest, we found that males were less inclined to accept food as they age, and that males who did accept food had fewer and shorter interactions with brood as they aged. This negative association between helping effort and age suggests that males' investments in helping diminish as they get older.

However, there was no association between male reproductive maturation and expression of helping behaviour.

Some helping behaviours observed in hymenopteran males, such as nest thermoregulation and defence, are likely to be mutually beneficial rather than truly altruistic (Cameron, 1986, Lucas & Field, 2011). Food-distribution behaviour observed in male *P. lanio* of this study is also likely to be mutually beneficial to both adult males and the brood, if the act of helping means females are more willing to give food to helping-males. For example, in this study (pers. obs.) and in other *Polistes* species (Cameron, 1986), it has been noted that food balls masticated by male wasps appear to decrease in size before they are transferred to the brood. Males may take some food for themselves, and distribute any remaining food to brood (Cameron, 1986; Giannotti, 2004; Sen & Gadagkar, 2006). However, it is important to realise that female workers may also derive some nutrition during food handling before provisioning brood, and hence female behaviours are not always truly altruistic either (Hunt, 2007). The degree to which males benefit nutritionally from helping, and the impact of their helping effort on brood productivity remain unknown.

Helping effort by males decreased with age on natal nests. This was evident from both the acceptance of food and subsequent distribution of food to brood. Importantly, rejection of food by older males suggests that males are not primarily acting selfishly (i.e. for self-nutrition), and only feeding brood secondarily. Moreover, although the highest fitness payoff may change to mating and producing daughters, males did not always exploit food by taking foraging and not distributing it to food. In animals with temporary helpers, helping effort is known to decrease shortly before dispersal (Zöttl et al., 2013a). The consistent age-dependent effects on helping effort suggest that male helping may be a modular temporal behavioural phenotype which is fixed in sequence (West-Eberhard, 1989), such that once a male passes through this behavioural transition they may not be able to revert back to being a helper. The reduced investment in helping with age supports the idea that there is a behavioural switch in males, with young helpers investing in indirect fitness (whilst gradually maturing), and sexually mature males investing in direct fitness (by dispersing putatively to mate – Chapter 5).

One possible regulatory mechanism for male helping behaviour could be physiological changes associated with reproductive maturation. However, we found no support for this, state of sexual maturation did not explain investment in helping behaviours. One possible explanation is that male helping behaviour is more complex than original hypothesised. Juvenile hormone is known to regulate maturation and behaviour across the insects (Hartfelder, 2000). Males of *P. lanio* were found to depart earlier from natal nests when given an application of the juvenile hormone analog methoprene (Chapter 5). If juvenile hormone is responsible for the regulation of behavioural repertoires in males, beyond just maturation and dispersal, then there could be a time-lag between actual dispersal and behavioural transitions.

A time-lag between responses may explain why some males were still present on natal nests, with little investment performed in helping effort. Given the effect of juvenile hormone in increasing female aggression with queen-like behaviours in *Polistes* paper wasps (Barth et al., 1975; Röseler et al., 1984; Giray et al., 2005; Tibbetts & Izzo, 2009), it could be expected also to have reproductively linked behavioural effects in *Polistes* males. Future work should examine whether topical applications of methoprene influence expression of helping behaviour in males, irrespective of age.

Observations across the Hymenoptera have assessed that males are often inferior helpers in comparison to females, for example that males take longer to masticate food balls and feed fewer larvae per solid food load (Harrison, 1987; Hunt & Noonan, 1979; Cameron, 1986; Sen & Gadagkar, 2006). However, these studies compare old female workers (who also forage) with young males. Comparisons of relative helping efforts of males and females require comparable experiments on the feeding behaviours of newly emerged young females, which (like males) do not typically forage (Topoff & Mirenda, 1978; Keeping, 1992; Giray et al., 2005; de Oliveira Torres et al., 2013). Inferior helping behaviour by males may be attributed to morphological and behavioural limitations, or more selfish acts. In exploring the difference between whether male helping is limited or reduced by selfishness, tests need to reveal the capabilities of males versus females in performing tasks.

Male helping behaviour may be selected for if it helps maximise a male's inclusive fitness (Sen & Gadagkar, 2006). In Chapter 3, we found that male and female brood shared the same mother in *P. lanio* nests, meaning males would have the maximum relatedness to siblings (life-for-life, $r = 0.5$), and hence there is likely an indirect fitness component to helping for males, as they may help raise full-siblings (similar shared maternity between male offspring and siblings was also found in the sister species *P. canadensis*, Chapter 2). Helping raise sibling nestmates could therefore help maximise inclusive fitness by investing in 'the best of the current situation', until direct fitness opportunities are available, e.g. through dispersal or mate availability. Although inbreeding could favour male helping in some situations (Davies & Gardner, 2014), we found no evidence of inbreeding in *P. lanio* (in the same Trinidadian population $F_{IS} = -0.02 \pm 0.02$, Chapter 3). Both Sen & Gadagkar (2006) and this study detected that males may preferentially feed large larvae over smaller larvae, and this may be because large larvae are more likely to survive to pupation, and hence provide a better return to indirect fitness investment. Large larvae are also less likely to be selected for intracolonial cannibalism (Polis, 1981; Schmickl & Crailsheim, 2001). Intracolonial cannibalism could be a strong selective pressure in helping for *Polistes* males; because cannibalism is more common towards the end of the nesting cycle, and this is a time when most males are produced and present on the natal nest, meaning they are present during this time of upheaval (Hunt, 1991; Turillazzi & Cervo, 1994). Alternatively, investment in large larvae may simply reflect the

higher developmental nutritional demands of the larger larvae (Wilson, 1971; Hellmich & Rothenbuhler, 1986).

The findings of this chapter suggest that helping may be a male trait under selection. Males contribute to the nest group in a similar way to females (albeit, likely at a lower intensity), they have the potential to gain indirect fitness benefits from doing so, and helping behaviour appears to be a fixed phenotypic component of male life history rather than being randomly performed by chance. In contrast to female helping behaviour, explaining the evolutionary origins of male helping behaviour is challenging. Female helping behaviour is likely derived from redirection of ancestral maternal care towards siblings (West-Eberhard, 1987; Linksvayer & Wade, 2005; Toth et al., 2007). However, ancestral paternal care is largely absent in the Hymenoptera, and therefore male alloparental helping cannot derive from this pathway (Andersson, 1984; Bourke & Franks, 1995; Queller, 1997). Male helping behaviour, therefore, could be an evolutionary innovation (Müller & Wagner, 1991). However, a simpler evolutionary pathway is through mutually beneficial actions. Male helping is likely to benefit both the adult male (by self-feeding) and brood (Cameron, 1986; Giannotti, 2004; Sen & Gadagkar, 2006; Hunt, 2007). Male helping may have originated as self-feeding, with food taken from maternal or alloparental female foragers, and excess food given to brood-kin by males; increased indirect fitness and selecting for helping. Once dispersal and mating opportunities are available, males that remained helpers on the nest would receive lower fitness than those that departed to mate, hence there would be selection for a fixed phenotypic transition between two clear temporal phases: a helping phase and a reproductive phase. In male life history, this transition would be fixed, once individuals transition through to a reproductive phase there is no plasticity allowing a return to a helping phase (West-Eberhard, 1989). Investigation into male temporal phenotypes may be the next step in uncovering traits involved and regulation of male helping behaviour. Future work may also address the question of whether male helping is underpinned by the same molecular basis as female helping behaviour, or whether male helping represents a novel phenotype (Toth et al., 2010; Ferreira et al., 2013; Sumner, 2013).

6.6. Appendix List

Associated appendixes: Appendix VI – male wing length correlates with body size measurements; Appendix VII – male eye colouration with age; Appendix IX – alternative zero-inflation models.

Chapter 7. General Discussion

7.1. Overview

In this thesis, we explore male hymenopteran behaviour at the start of adulthood (Chapter 5 & 6), and by extension the inclusive fitness maximisation (Chapter 2 & 3) and lifespan (Chapter 4) of females in two social wasps *Polistes canadensis* and *Polistes lanio*. Male helping, in which mostly natal males have been observed to contribute some way to the nest (such as distribution of food to brood), has been sporadically reported across the Hymenoptera but rarely explored in detail (see Chapter 1, Table 1.1). We aimed to investigate male helping behaviour by asking the following questions: is there an indirect fitness benefit to helping; are male fitness returns potentially assured by long-lived females; are males delayed dispersers, what regulates departure; and is helping a normal part of male behaviour, or an abnormal occurrence. Here, we present our main findings and discuss them within the context of male helping.

7.2. Summary of main findings

7.2.1. Nests consist of highly related siblings

Chapter 2 & 3 summary: Using newly discovered SNP loci in the paper wasps *P. canadensis* and *P. lanio*, we developed a set of genetic-markers to investigate relatedness and reproductive skew in newly-established foundress nests and established post-emergence nests (the latter producing males). We found that in *P. canadensis* nests are typically formed by either renesting mothers and their daughters (eusocial establishment) or half- and full-sisters (semi-social establishment). Foundress nests possessed skewed reproduction, in that usually a single female monopolised egglaying, and this skew was extended in post-emergence nests of *P. canadensis* and *P. lanio*; with dominant females laying most of the female and male brood. This monopoly meant that both female and male siblings had (assuming equal sex ratios) an average relatedness to brood on the nest of $r = 0.5$. A maximum average relatedness of $r = 0.5$ to siblings (under equal sex ratios), means that helping is likely realised (via indirect fitness) for both female and male offspring, so long as fitness is assured via long-surviving nests. Multiple mating, usually with a single additional male, was found in reproducing females of both species. However, multiple mating did not seem to effect overall relatedness in brood, which was constantly high enough that on average offspring shared parentage (brothers shared the same mother as female and male offspring).

Male helping may generate indirect fitness: High relatedness between offspring in post-emergence nests means that male acts of helping on the nest have a potential indirect fitness component. Brothers have the highest relatedness attainable to siblings of (life-for-life) $r = 0.5$. Under equal sex ratios, $r = 0.5$ is the same relatedness of sisters to sibling brood on the nest; by this argument males may mimic the helping behaviour of females to exploit these same indirect components of inclusive fitness (though males likely have higher costs to helping than females, which are morphologically adapted for the role – Starr, 1984a; Starr, 1985a). Male behaviour could be either (weakly-) altruistic, of mutual benefit, or a by-product of selfish actions. High relatedness between the actor and recipient allows altruistic behaviour to be realised, as Hamilton's (1964) rule states that the benefit and relatedness to the recipient must outweigh the cost of the behaviour. Behaviour that benefits the recipient, that is of mutual benefit for the actor or by-product of selfish actions, does not require high relatedness to the recipient; but because all three types of interactions (including altruism) are viable then we may expect more chances for male behaviour to evolve. We do not yet fully understand the costs of helping for males, but expect that is it low and somehow benefits the male also (such as if a male also eats a proportion of food it distributes) (Lucas & Field, 2013). In the *Polistes* paper wasps, social environments are dynamic, and therefore fitness payoffs may change quickly throughout an individual's lifespan; for example, there can be a turnover of queens, fighting among potentially reproductive female workers, catastrophic nest attacks from large predators, and social and brood parasites of the nest (Pardi, 1948; Strassmann et al., 1988; Strassmann et al., 1989; Judd, 1998; Hughes et al., 2003; Cervo, 2006; Jandt et al., 2014). If the costs and benefits of helping fluctuates for males, then interaction outcomes in terms of fitness may vary between being altruistic and mutually beneficial. For example, if in times of reduced forage, males change how much food they intake and how much they give to brood (being either more to themselves or brood); whether a threshold such as this exists is unknown. We also detected multiple mating in females, and this is of interest because it is expected to be rare in *Polistes* (Strassmann, 2001). Irrespectively, mother mate number does not change male relatedness to siblings: to brothers, males are haploid copy of the mother's chromosomes, and no fathers are involved ($r = 0.5$); to sisters, males only share the mother's chromosome and not the fathers (relatedness coefficient, $r = 0.25$; life-for-life, $r = 0.5$). Therefore, if male helping is reliant on the degree of recipient relatedness outweighing the cost, then it is resistant to the dilution of multiple mating unlike helping in females (Strassmann, 2001). However, average mate numbers for reproducing females (in this thesis: *P. canadensis* 1.22; *P. lanio* 1.15) were not has high as found in some complex eusocial species such as common yellowjacket wasps *Vespula vulgaris* (1.90) and honeybees *Apis mellifera* (12.40) (reviewed in Strassmann, 2001). A low mate number means only a select few males likely mate with a successful queen, despite equal sex ratios of reproductives, because every mated gyne will not become a dominant reproductive on a new nest (e.g. in *P. canadensis* foundress nests in this study, most females had mature eggs in their reproductive tract and were likely mated). Indirect fitness through

acts of helping in natal males may therefore be the only gains towards inclusive fitness some males receive. However, given males always disperse to mate, the chance of reproducing and having daughters likely always outweighs acts of helping. Further studies are needed to fully understand the relationship between the costs and benefits of male helping, and how relatedness may influence evolutionary pathways.

7.2.2. Females are long-lived, male helping may be assured

Chapter 4 summary: We tested whether in the absence of extrinsic morality risk (i.e. using captive wasps), whether longevity patterns differed in female *P. canadensis*. Captive females had relatively long average lifespans for Hymenoptera, just slightly shorter than most bee and ant queens, though variation existed between groups. In wild *Polistes*, foraging workers usually have shorter lifespans than the dominant egglayer (reviewed in Chapter 4, Table 4.6), given our results all individuals likely have the potential to live similar lifespans when extrinsic morality risk is reduced.

Fitness returns are potentially assured by long-lived females: Males in the Hymenoptera are relatively short-lived in comparison to their female counterparts (Heinze, 2016). In addition, males usually leave the nest shortly after adult emergence (Hamilton, 1972; Jaffé et al., 2009; Johnstone et al., 2012). The male's short presence on the natal nest means potential helping males only influence a small proportion of the brood's development period. In *P. lanio*, males only stayed on the natal nest up to seven days (Chapter 5). However, in its sister species *P. canadensis*, larval development from a small larva to pupa can take a mean of 25 days; further, large larva (which received the most acts of male helping effort in food distribution, Chapter 6) can take up to 12 days to reach the self-sufficient pupation period (in terms of feeding) (Bell, 2016). Therefore, even if a male helped each day of their time on the natal nest (Chapter 6 suggests on the contrary male helping on the nest diminishes with age), then individual male helping would still be under half the development time of a large larvae to pupae development. Female Hymenoptera are 'life insurers', they require other helping females to have overlapping lifespans to overcome the risks of off nest predation whilst they forage; fitness returns are therefore assured (Gadagkar, 1990; Queller & Strassmann, 1998). We suggest that males also require such fitness assurance, their short temporary helping effort is likely supplemented heavily by female helping (and maybe by other males). Because here *P. canadensis* females demonstrate long lifespans, they have the potential to stick around on the nest long after a male has departed, and finish the job of raising brood.

7.2.3. Males are delayed dispersers

Chapter 5 summary: In studies of *P. lanio* dispersal behaviour, males were found to disperse at a median age of three days old, with some variation in dispersal times dependant on the site location of the nest. Males sexually matured on the nest, and dispersed males caught off nests around sites had more signs of sexual maturity than those males still nesting. Through applications of the juvenile hormone (JH) analogue methoprene, we found that JH likely regulates dispersal (males departed sooner given methoprene applications) and that JH also likely regulates sexual development (males given methoprene applications had accelerated development of the accessory glands). Therefore, ultimate dispersal is likely paced by internal increasing JH titers.

Males delay dispersal until sexual maturation: delayed dispersal by immature males sets the stage for helping behaviour to be realised. Given that this 'obligatory pre-mating period' on the natal nest can be observed in many other social Hymenoptera, it may be a trait associated with social nest living that males stay temporarily on the natal nest after emergence (Hagen, 1953; Gobbi, 1975 in Giannotti, 2004; Ruttner, 1976; Duchateau & Mariën, 1995; Tasei et al., 1998; Rhodes, 2002; Baer, 2003; Poidatz et al., 2017). As non-nesting dispersed males had significantly more signs of sexual maturity, natal dispersal seems reliant on internal development. However, there was some variation in dispersal times between sites, and measures of nest characteristics (such as size) did not account for this variation. Whether site location had internal or external influences on dispersal is unknown, such as if some sites had better foraging (e.g. improving 'internal' maturation) or reproductive opportunities (e.g. 'external' lekking sites or receptive females) is unknown. Though males did sometimes receive aggression when we placed them back on the nest during marking, in those males that did not immediately leave the nest (after a 5 min observation period) aggression did not significantly influence dispersal age; suggesting that dispersal induced by aggression was limited to the abnormal situation of being marked. Although this measure of aggression is limited, it would initially reject the claim that males are kicked off the nest by females; rather it would seem that males leave the nest when they are ready on their own will. However, if female aggression was associated with male maturation (i.e. only when males reached maturity to avoid inbreeding – Pusey, 1987; Pusey & Wolf, 1996), then our observations would not be able to detect this. Irrespective of the final causation of departure, males appear to have two distinct life history phases, of being an immature social nestmate; and a dispersed mature sexual (Chapter 1, Figure 1.2). We suggest that this first immature social phase is a prime candidate for observing potential helping behaviour (as seen in Chapter 6), because males cannot mate therefore they may perform helping to maximise inclusive fitness – if the act of helping does not decrease future fitness potential (i.e. helping is not costly to the male).

Dispersal is regulated by juvenile hormone: in the female Hymenoptera, JH is behavioural pacemaker and gonadotropic regulator (Barth et al., 1975; Röseler et al., 1984; Robinson, 1987; Robinson et al., 1991; O'Donnell & Jeanne, 1993; Giray et al., 2005; Bloch et al., 2000; Hartfelder, 2000; Shorter & Tibbetts, 2009; Tibbetts & Izzo, 2009; Smith et al., 2013). Though we cannot be sure if JH in *P. lanio* males is a behavioural pacemaker or gonadotropic regulator (or both): for example, if JH causes behavioural changes, which then induce dispersal; or if it influences sexual development, which then causes dispersal. Whatever JH pathways influenced dispersal, it did have a profound effect, behavioural and developmental changes happened only two days (and immediately the next day in dispersal) after methoprene applications. The question arises, if JH is critically involved in dispersal behaviour, could it also regulate male helping behaviour? In female *Polistes*, JH upregulation is involved in both: age polytheism, in which nesting tasks are performed by younger females who then switch with upregulation to off-nest foraging tasks; and queen-like behaviour, in which females stop helping and become more aggressive to other nestmates (Shorter & Tibbetts, 2009; Tibbetts & Izzo, 2009). It is therefore not a huge leap to suggest that JH might have shared properties in both males and females. If males and females share similar hormonal mechanisms that control behaviour, then there may also be other hormonal and therefore genetic shared pathways. Future research may wish to employ RNA-seq methods, in exploring shared expressions between young males and females, and that of mature individuals committed to roles (such as reproductive males and females, or worker females) (Sumner et al., 2006)

7.2.4. Males help

Chapter 6 summary: In performing field trials on natal *P. lanio* males, in which we experimentally offered males a food source each day till departure; we discovered that males contribute to food distribution by feeding brood our offered food, but that this effort diminishes with age. Males also were observed taking food from foraging females, and performing similar food distribution behaviour. Effort to help diminished in both initial acts to accept the food, and if they accepted the food in acts of male-cell interactions (a proxy for food distribution). However, we found no association with helping effort and variance in reproductive maturity.

Males help, but helping effort deceases with age: As suggested by reports of male helping across the Hymenoptera (Chapter 1, Table 1.1), and confirmed by our observations of males taking and distributing food offered through experimental trials and from returning foraging females, male helping seems to be a normal part of male life history in some species and not an anomaly. We are not however suggesting that males contribute to societies on the same level as worker caste females, though they share similar behavioural actions in helping, male helping is likely

performed less efficiently (such as number of brood fed – Sen & Gadagkar, 2006). The question arising from is how much males contribute to brood development, and whether it is significant or not. We suspect that males cannot support nests on their own: first, they never forage food themselves; second, previous removal trials looking at male defence behaviour in the wasp *Microstigmus nigrophthalmus* (in which all females were removed and males left alone with brood), found that although males alone did increase the survival of brood it was not as good as female defence (Lucas & Field, 2011). More likely, is that males provide an additional service on-top of female efforts. For example, in the rare case of the paternal caring hymenopteran male *Trypoxylon politum*, male defence allows females to devote more time to foraging rather than defend the brood themselves (Brockmann & Grafen, 1989). If food distribution is takes time and energy (e.g. in handling, masticating, and finding hungry larvae), then females could reduce time spent performing this behaviour and spend more time foraging (a behaviour which males cannot perform). An emerging theme is the cost and benefit of male helping to the both the actor and recipient, which may be resolved through future experimental manipulations of removing female and males on the nest and observing brood development.

7.3. Thesis conclusion

We have demonstrated that hymenopteran males do help under certain conditions, though these acts are likely far from the altruistic actions of their worker caste sisters. Males could be young temporary helpers, and old reproductives. Referring to Hölldobler & Wilson's (1994) description of males being parasitic "flying sperm-bearing missiles constructed only for the instant of contact and ejaculation", does this still hold true? In most instances, probably yes. However, here we argue that there is more to male behaviour in some Hymenoptera, and maybe these 'flying sperm' start off with more humble origins as a semi-contributing helping member to societies; before at least they start their sexual escapades later in life

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Other:

(Moreno et al., in prep) – *Polistes lanio* transcriptome data.

Appendix

Appendix I

Appendix Table 1. Genotype sample list for Chapter 2.

ID	Genotyping Method	Genotyped Samples				
		Number of Female Adults Genotyped	Number of Female Eggs Genotyped	Number of Female Pupae Genotyped	Number of Male Eggs Genotyped	Number of Male Pupae Genotyped
FA #01	SNPs	5	3	0	0	0
FA #02	SNPs	4	8	0	0	0
FA #03	SNPs	9	8	0	0	0
FA #04	SNPs	4	8	0	0	0
FA #05	SNPs	6	7	0	0	0
FA #06	SNPs	4	8	0	0	0
FA #07	SNPs	8	7	0	0	0
FA #08	SNPs	12	8	0	0	0
FA #09	SNPs	9	7	0	0	0
FA #10	SNPs	4	8	0	0	0
Mean ± SE FA (SNPs)	SNPs	6.50 ± 0.90	7.20 ± 0.49	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Post-emergence #01	SNPs	2	2	6	3	4
Post-emergence #02	SNPs	2	3	5	2	5
Post-emergence #03	SNPs	2	1	5	4	5
Post-emergence #04	SNPs	2	0	5	5	5
Post-emergence #05	SNPs	2	4	5	1	5
Post-emergence #06	SNPs	2	3	5	2	0
Post-emergence #07	SNPs	2	4	5	1	0
Post-emergence #08	SNPs	2	1	5	4	5
Post-emergence #09	SNPs	2	1	5	4	5
Post-emergence #10	SNPs	2	3	5	2	5
Mean ± SE Post- (SNPs)	SNPs	2.00 ± 0.00	2.20 ± 0.44	5.10 ± 0.10	2.80 ± 0.44	3.90 ± 0.657
Post-emergence #11	*Microsats.*	0	0	5	0	0
Post-emergence #12	*Microsats.*	0	0	5	0	0
Post-emergence #13	*Microsats.*	0	0	5	0	0
Post-emergence #14	*Microsats.*	0	0	5	0	0
Post-emergence #15	*Microsats.*	0	0	5	0	0
Post-emergence #16	*Microsats.*	0	0	5	0	0
Post-emergence #17	*Microsats.*	0	0	5	0	0
Post-emergence #18	*Microsats.*	0	0	5	0	0
Post-emergence #19	*Microsats.*	0	0	5	0	0
Post-emergence #20	*Microsats.*	0	0	5	0	0
Post-emergence #21	*Microsats.*	0	0	5	0	0
Post-emergence #22	*Microsats.*	0	0	5	0	0
Post-emergence #23	*Microsats.*	0	0	5	0	0
Post-emergence #24	*Microsats.*	0	0	5	0	0
Post-emergence #25	*Microsats.*	0	0	5	0	0
Post-emergence #26	*Microsats.*	0	0	5	0	0
Post-emergence #27	*Microsats.*	0	0	5	0	0
Post-emergence #28	*Microsats.*	0	0	5	0	0
Post-emergence #29	*Microsats.*	0	0	5	0	0
Post-emergence #30	*Microsats.*	0	0	5	0	0
Post-emergence #31	*Microsats.*	0	0	5	0	0
Post-emergence #32	*Microsats.*	0	0	5	0	0
Post-emergence #33	*Microsats.*	0	0	5	0	0
Post-emergence #34	*Microsats.*	0	0	5	0	0
Mean ± SE Post- (*Microsats.*)	*Microsats.*	0.00 ± 0.00	0.00 ± 0.00	5.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Appendix II

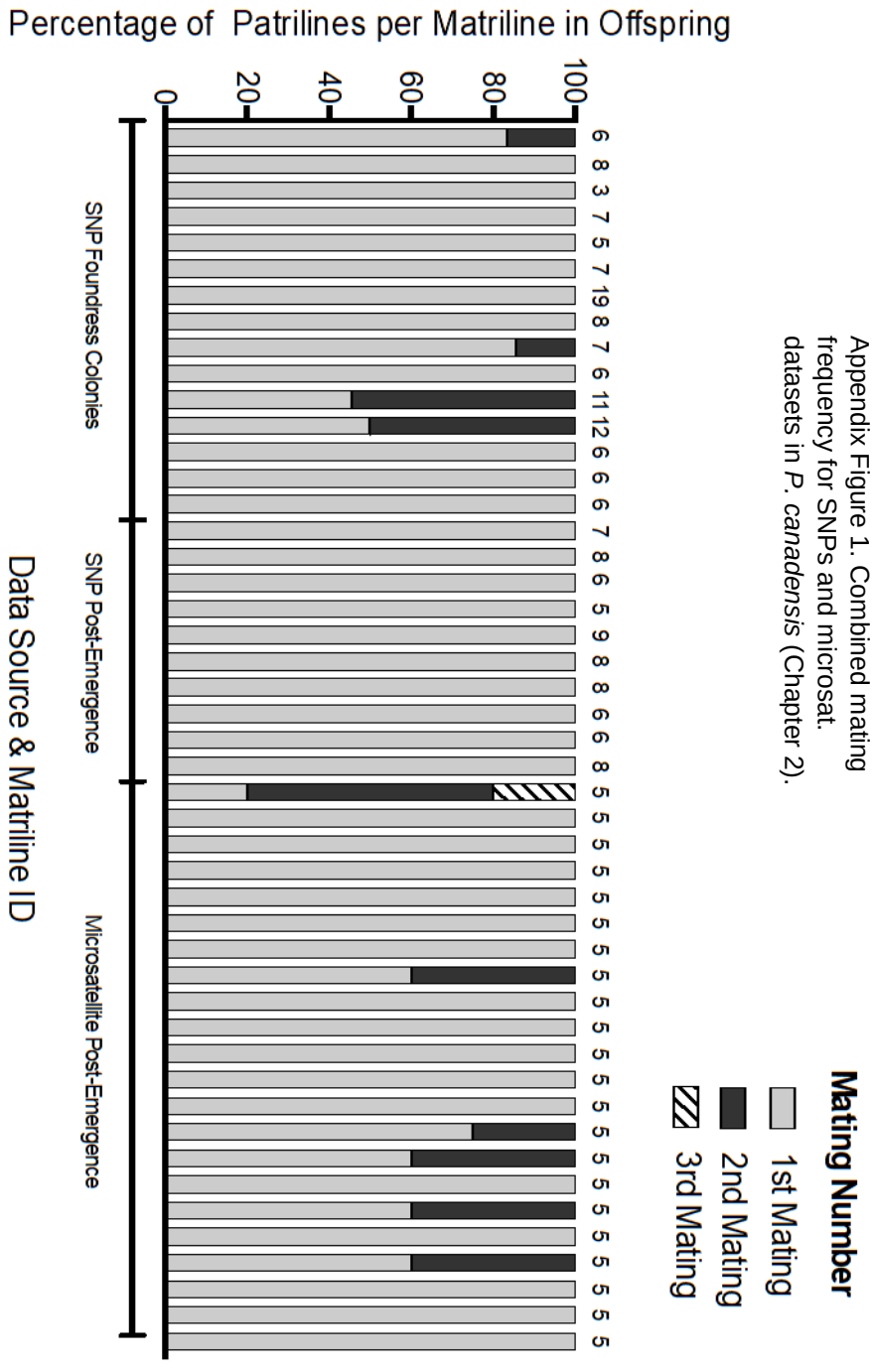
Appendix Table 2. SNP position, count, coverage, and frequency – with validation for the *P. canadensis* and *P. lanio* study populations (Chapters 2 & 3).

SNP #	Position	Alleles	Count	Cov	Frq	[SNP] and surrounding 50 bases	Val. *Pcan*	Val. *Plan*
Polistes SNP #1	scaffold_1:1273098	C/T	605	1226	49.3	TGGTAGAAACAGTATATTCAGGCAAATTGTTTTCAATCATCTTGTTGCTTAGATTC[T/C]TAAGCCCATATAATCTCCTAAAGAAGGATATATAGGGCCAGATGGAGAAGAATCA	Yes	Yes
Polistes SNP #2	scaffold_2:374033	A/G	447	835	53.5	CTAAGGGATAAGCATCGCCTTCCTTTGCCCTTACAGGATTACTCGGGAA[A/G]TGTGTTTCTATTATATCTCCAGGTTTCATATTTCTTGTCGCTAAAATATACTTC	Yes	Yes
Polistes SNP #3	scaffold_4:2247489	C/T	440	1008	43.7	AGTCGTTACGGCTTGCTTTTATTTTTTCTTACTTTTCGTTTTTCTTTCCTTCCTTCTTCTTCTTTCGTTTATTTTCTTTTGTTATCTTTTGT		
Polistes SNP #4	scaffold_4:2246609	G/A	384	951	40.4	GACGAGGGGACGAGGAGGGTGCTGGGGACGAGGGGTGATCAGGGTGATCAGGG[G/A]ATGATGTATGGGGAAAATGGATGACGATGAAGATGCTTGAAGATGACGGAAC		
Polistes SNP #5	scaffold_5:582041	A/C	583	2000	29.2	ACTTTACGGCCAAGCAATTTGGTTTTAAAGATGTGCAATTAAATGCATTTAATAGAGGTACCTGTATTGTGCCTTAATGGG	Yes	Yes
Polistes SNP #6	scaffold_5:573991	T/C	571	1327	43.0	GACCGCTTTCGAGGTTTTGAAAATCTCGAAGAACTCGACATCTCACAATCCA[T/C]TATCGGATGCTTTCGTTATCTTGTTAAGTCCATTAAGCTCCTTAGAATTTTTGG		
Polistes SNP #7	scaffold_5:159166	T/C	444	928	47.8	GAACAAAATATCCAACTTGGACAATTGACTATAGGATGCTATACGATTACCT[T/C]TTAAAACCGTAAAGTGGTATTTAACAGTTGTCGTATAACTTGGTCCTGATTAC		
Polistes SNP #8	scaffold_6:138579	C/T	419	1543	27.2	AAATAAACGAGACGCGGTGTTCGATGGGACAGCACTTTTTCACGAATCACCGA[C/T]GACGATTCCACGGAAACGACAAGTGACCGTAACTATTCACAGATTTGGAAGTGTTC		
Polistes SNP #9	scaffold_6:954600	T/C	321	1385	23.2	ATTATCTTTTTATATTTATCATAAAGGTTACCACAATAGAGAGACCCGTTTA[T/C]GAAATAATTTTATATTCGGAAATCTAAACAGACGATATTTTATTTTCTCATC	Yes	Yes
Polistes SNP #10	scaffold_6:956102	C/T	240	976	24.6	GCGTGACACACACCTAACTACATAGGTATATTTATACAGCACTATGCTATATTATT[C/T]TACACAACATGATAATTTTCGTTTTTTTTACGGATATTCATGACTTATCCTCGAA	Yes	Yes
Polistes SNP #11	scaffold_6:112777	T/C	733	949	77.2	TTTAGGAAGACGAAAACGTGGCACCCAATACGATTTCGTTGAGTTT[T/C]GTAAAAGGTGGCCGTGTTGATCATAGACTTAAAAGGGATGCAACAATCCGAAAGCC	Yes	Yes
Polistes SNP #12	scaffold_6:113032	G/A	459	814	56.4	GTCTTCTTCAATACTTGGACACTGGCCGTTGGTTCTTATCTGTTTATAACGATGA[G/A]CTTCAACGTATAAGGTAACGTCGGTCGTCACCGAAGCTGAAGGAGTTTCCACT	Yes	Yes
Polistes SNP #13	scaffold_7:1024471	T/C	1037	2035	51.0	CTTCTTCTTGATATTGTTCAACTTGCATAGAGTGCCTTAGATAAAGGTGCCTTAGATTCCCAATAGACTTTATCGGTCTCTTGTATTGTT		Yes
Polistes SNP #14	scaffold_7:1062289	G/A	588	1327	44.3	TTTTTTTTTATGTGCACGTGGAGCGATTCTTATGTAAAGATAATATTAATAATATGTACTACACGTCGTAGGTAATTATCGAATGGTAAGAAACA		Yes
Polistes SNP #15	scaffold_7:1023709	C/G	385	1275	30.2	ATGGCCGGAGAGTTGCAGCAAAGATGTTTCGTTCTTGTATATAATAATTT[G/A]AATTGCGTAGATAAGATACCTTTGCTCTTGTATCTTCAAAGCTTACTGGCATATT	Yes	Yes
Polistes SNP #16	scaffold_8:1197598	G/T	773	1060	72.9	AGGAACTTGTTGGCGTTGGTAGTCCATTGAGAGAGGCCTCACGAGACCGCGAAGT[G/T]CGTGTGTGGTCGCACGACCATCGAATCCGTCGTCGGCTTGCTGTTGTTCAGTCA	Yes	Yes
Polistes SNP #17	scaffold_10:169607	T/C	181	765	23.7	TTTTCTTATCATCCTCTTCGTCGTCATCATCATCAATGGCTC[T/C]TTTAACCATTCTTCTGGTGATACTATCAAACTTCTTTACGTTGC		
Polistes SNP #18	scaffold_11:288804	G/A	705	1943	36.3	CGTAATGGATGGATGTGGGAAATAATTTTCACGAGGAAATATGATGTATCAAACGGAAA[G/A]TCCCGAAGATAAGGAAGCAGCGATCGTAAGAAGCAGAAATC	Yes	Yes
Polistes SNP #19	scaffold_12:1443422	A/G	7395	16794	44.0	GATCTTCTTTAATGTGCAAAAATGATTTTAGTACCTCTTCCAATAGGTTCTCCCATTA[G/T]CAGGACGAACTATGAAAGAACCACCGGCATGAGGATTCCCATAAATACTGTTCA	Yes	Yes
Polistes SNP #20	scaffold_12:361643	G/A	367	1023	35.9	GACCATGATTTAAATCTTATTATAGTGAATCTGGTTACATATTTATATAATAATTT[G/A]AATTGCGTAGATAAGATACCTTTGCTCTTGTATCTTCAAAGCTTACTGGCATATT		
Polistes SNP #21	scaffold_12:577710	C/A	283	766	36.9	GCCTGTAGACAAAAAGTGGTCTGAAGAATTTGGCCTCGAGATCATA[C/A]GATTCGTTTTTATTTTCCTCAGAAATCGATCTTGGAATTCGTCGAAGCGAATGT	Yes	Yes
Polistes SNP #22	scaffold_13:324230	A/G	269	848	31.7	CGGTGGCAATCTATTGTTGAGGGATAAATTTGTGTCAAAGTATATGGTGGTAAATTA[A/G]GTGTGGTGGCAAACATAGCTTGGCCTAATCAATGGGTGATTATTGATCGGTTCGTTC		Yes
Polistes SNP #23	scaffold_14:842609	G/A	1772	4029	44.0	TATAAAATATTTGGGCCGTAGAAATGCGCTCTATGGTTCCCCAACAGTGACGAGC[G/A]CGTTTCGCGCTCAAATTTTACTTCCTTGGTTGCTGAATGATAATTGGACGCGAA		Yes
Polistes SNP #24	scaffold_14:1020816	A/G	1638	2669	61.4	GCACGCGATCATCATTTCACGGGGGGATAGCGTAGCGTTATGGAAGGTCGTCC[A/G]GAATACATTGGAAGTTGGTTGGGTGTTACGAAGGTCGGTTTCGTCGGTCGCTCTT	Yes	Yes
Polistes SNP #25	scaffold_14:702796	T/A	205	869	23.6	TTGATTTAAATAATGTATATATTTTTAACGTCACGCACCTGTTTA[T/A]CAAAACTACTATTTTTGTCACATGTGGCGTCATCTGTATGTCTGATCCAATTAAT		Yes
Polistes SNP #26	scaffold_16:1091583	A/G	345	843	40.9	ATTGTGACATAGCTGACCATATTCTTCTTCTTATGTAAAACTCAGCTGAAACCAGGTAAGAAAGTGTCGCTATCACCTTAGGAGGATGA		
Polistes SNP #27	scaffold_18:840171	A/G	618	1260	49.0	GTAGAATTATATAATTTTTTGAAAGAAGAAGGCACATTTTAAGGA[A/G]ATATATTAACGGAGAGTAAAAAACGAGATCCCCCATATTGCTAAATGTCCTT	Yes	Yes
Polistes SNP #28	scaffold_19:149510	T/C	497	910	54.6	CTTGGATCAAACAACATTCATCGGTATCAAAATTATTGGATGGTTGTGATTATCT[T/C]GGTGAATGGTTGGCCAATTTTTTTGGTATTACAAGGCTAAATATCAATTTGAA		
Polistes SNP #29	scaffold_19:357631	T/C	670	872	76.8	AAATAATCGAAGTACCAAAAGGACAGTGCATTATTTCTCGAGGAAGCCGAAGGTTTT[C/G]CATCGTGAATTTGCTAAAAGATTAGCGGCACGTATAGATTTCAAGCCA	Yes	Yes
Polistes SNP #30	scaffold_23906530	C/A	398	1509	26.4	TAGGATTCGGACGTATTTGTCGTCCAGTTCGCTTTCTAAAAACTTT[C/A]AGTTCTCGCCTTGCCAGGTAAAATATTGACGGCTATGGAAGTATCGAAGGGGTCA	Yes	Yes
Polistes SNP #31	scaffold_23906754	C/T	296	1049	28.2	TTTTCCGATTTTTGTTTCCAATTATTTCTTTCCAATGGATTGCCCACAGTTGCCATGAA[C/T]AAATTCTAAATTATAGGCCTGAATGAAGTATCGGTATAAAATCGCTTTTTCTTCT		
Polistes SNP #32	scaffold_23906883	G/A	510	1012	50.4	CTCGGATTTAAATTCGTCGACTCAGCTGTGCGATAATTTTTGATTCTTCTTCCA[G/A]TAAATTCGGATTCGATTAATTTCAGCTCGGTTTTTACCAGGTGTTACTTTATGTCAC	Yes	Yes
Polistes SNP #33	scaffold_23:4065557	T/G	553	963	57.4	CAATATATTGCGCCTGGATGGGCTATTTTTATTTATGAGACATCGTTTGAAAGGCTTCAAGTATTCCTCATCGACAATTTGTCAGAGAAGCTCAAAAGAAACCATATTTCGTGAATTGCGTCATATTACT		Yes
Polistes SNP #34	scaffold_26:251809	G/A	483	818	59.0	CATTTCTATGATCAAACTTGAAGTGCTTTATTAAGACATCGAAAGGCTTCAGTATG[G/A]CGGCCAGCTTTGAAGTGATCTATTCCTTCAGCAACACTTCGGAAATGCCCACTTG	Yes	Yes
Polistes SNP #35	scaffold_27:563258	G/A	18871	25795	73.2	GAAGATGCATCTCGAATGGAAGAAGTAGATTAAATATTCTTAACAAATTTTAAAG[G/A]TTATGGCATTATGTCATAGTATAGACAAAGACTGTTCAGATCAAAGAGAAATCT		
Polistes SNP #36	scaffold_30:597203	A/T	924	1412	65.4	GATCCTCTCGTGGCATAACTCGTTGCTAACTAGGAAATACGATTTAATTATTATA[A/T]TTATTAACCCCCCCTGCCTCTTCGTTTCTAATCTCATTATTTATTGAAACACGCGG	Yes	Yes
Polistes SNP #37	scaffold_30:590478	A/G	366	1134	32.3	ATTTTAATTTTTGTTTCATTCGTTTCTTCATTTGCGACGATCGTTAAAGAAAAATTTA[A/G]GCGTGTTTAGCGAGAGGAAAAAGTGTCGCTAATATTTTTATAGATTTCACGAAA	Yes	Yes
Polistes SNP #39	scaffold_31:443566	G/A	760	1571	48.4	TAATCTTTTGTGCACACCATGTCGTTTAAGATTTCAAGCTTTGCCAGAT[G/A]TTTCTTAAGAAACGCGTGTAAGCTAAATGCGAAGCAGTTAAGCTAAGTGTCATCACGTAA	Yes	Yes
Polistes SNP #40	scaffold_32:300980	C/T	574	780	73.6	TTCTTCGTGGCGTGAGCCTCAAAGAGCCCTGTCGAGACACCGACAAGGAACCCTC[C/T]TTGTGTTAATAACTTCGATGGTGCGGTTTACAGGTGTATGTGCTTCGTCCAAGTCA	Yes	Yes
Polistes SNP #41	scaffold_33:318773	A/G	294	1173	25.1	CGGTCCCGGTTGCTATCGTAATTATGAGCATTACAGTCTCTCCGGGCATCGTCGT[A/G]GCACGTGGTCCTGTTTCCGAATCTGCCGAATGACCTACGGACGACCCGGATA		
Polistes SNP #42	scaffold_35:935758	A/G	805	3271	24.6	GTGTTTCATTCTTTTTACGGTAGAGTGGGGGTAATGAAGGGTGCGAACGATGGAGA[A/G]GGTGGGAGAAGTTGAGAGCGCGGCGTAGATGGCGGTTGGTGTCATTAATGGGGGC	Yes	Yes
Polistes SNP #43	scaffold_35:486717	G/C	890	2730	32.6	AAAATACAACTTTCGAGGTCGTAGTATTAAGGAAAATTTATAGGTGTCGCATACCGAAAGGAAGAAGGATAAGTATCCAATGTTTCCATGATAATCGA		Yes
Polistes SNP #44	scaffold_35:487761	T/C	613	1195	51.3	TCGTATGATCGGTAAAATTTTTAATAGCAATTGAATTTGAATTATAATCGGACTTCTGAAATCGTAAAATCCGAAAATATTTC		
Polistes SNP #45	scaffold_40:54991	G/A	178	765	23.3	ATTGGTCAGCGGAAGAAAAAAAGAACACAACGTGAGGCTAGGGAATTTCTTTG[G/A]AATGAGATGGGACAAATGGTGAATCGGCGCTTCCAGGGACAAAATACCCATAAT	Yes	Yes
Polistes SNP #46	scaffold_42:223910	G/A	1316	2011	65.4	ATTTGTTCGTAAAATAAGTCGAAATTCCGAAACAATTGAAATATTCGGTTGA[G/A]GTGAGTTACATGGAGATTTATTGCGAGAGATGTTAGGGATTTGTTAAATCCAAAG		
Polistes SNP #47	scaffold_47:62723	G/A	609	2072	29.4	GAAAAATCATTCTCAACGATAGTATAGTGAGGATTTAAATACGGAGTTTACGGAAAGTTATCGTAG[G/A]TTTTTGAAATTTGTTCGGGAAAGTATCTATGTTTCGTGAATTCGGCTT	Yes	
Polistes SNP #48	scaffold_52:853973	G/C	401	1000	40.1	GCCTCCGAGAAGGGGGGTTGAATTGAAAAGAAAAAAACCCAAAAATATAAAAATAGA[G/C]AACTCTCCTCATCCTAACAAAAAAAACACTTCCTTCCTTCTTCTATTTTTCTTTT		
Polistes SNP #49	scaffold_52:853872	G/A	195	859	22.7	CTTCCTCTTCCGTAACAAGTATATTATTATCCTTCTTCCTTTAAAATCACAATGA[T/G/A]ACCAGAGAGAAGGGAATATTATATCCTTGAAACAAACGGGTGTTGTTTGGCTCCGAG		
Polistes SNP #50	scaffold_55:332517	C/T	1178	2388	49.3	TAGCCGTGTCGCTATCTCGGAAAAGATTTAGATCTTTACGGGAACGACTTGAGCAACGA[C/T]ATAGACGTCGTGAATTCATTAAGAGGACAAATTCGAGGATATCACCGGCTTGAGC		

Polistes SNP #51	scaffold_65:598531	G/A	459	1223	37.5	ATAAAGAAGATCGTGAGAGACAGGAACGCAAGGAACGAGAGGAAAGGGAAAGAAGG[G/A]GAAAAGGATGAGGAACGTAAGGAAAGGGAGAGATTAGACAAGGAACGTCAGGAG	Yes	Yes
Polistes SNP #52	scaffold_68:676431	T/G	552	1486	37.1	CTAAATTGATGGTCCAATTATCTCAATCTCAAGATGATGAAATCGGTGATGGTAC[T/G]ACTGGAGTAGTTGTATTAGCTGGAGCATTGTTAGAGCAAGCCGAACAATTATTA		Yes
Polistes SNP #53	scaffold_75:276569	G/A	1555	2032	76.5	TTGTTTAAATTCTCCTCACGTTGTTGTTAATTTTGTTAAAGTATGTGGATGACTA[G/A]AACAATCCTTGCCTAAAAGGATTGCTGTCACTTGACGGTCTTTGTTGTTCAACA		
Polistes SNP #54	scaffold_75:226680	A/G	283	1203	23.5	GTTTTTCTGGAGCTGGCAAATGAGATGGTACTAAATTTTCTTCTATAGGTGGCAT[A/G]GAACTCGATCTTTTGTGTTATATCCTCGATTGCGTTTAAAATAGCCGGTGCTTCG	Yes	Yes
Polistes SNP #55	scaffold_75:275026	T/G	560	816	68.6	AGCAATGATAACGTTTTAACTGAAACATTAGTATTAAAAACTGAAAAATTTGCCT[T/G]ACTTGGCAATCCGTTAAAGAAGGTCCTTGCTGAGTGGAAGCCTTCAATACTCGG		Yes
Polistes SNP #56	scaffold_79:338045	C/T	353	1009	35.0	GGGTTGGAAAAACGGAAGAAGATTTGAAGAAAGAAGGAATCAATTACAAGGTCGG[C/T]AAGTTCCCGTTCCTTGCCAATTCCCGAGCGAAAACGAATCTCGAAGCTGAAGGT		Yes
Polistes SNP #57	scaffold_81:561665	C/T	312	1261	24.7	GTTTGTAAAAGTTCGCACATAGGATTTTTATGTAATTAAAATGGTAATATTATAAA[C/T]TAAGCTTGAAAATTCGCGCCCTTTTTAATCGCCAATCCGCGTATTATGATCCTT		Yes
Polistes SNP #58	scaffold_94:477868	A/T	735	1027	71.6	ATAAATAATTATTAATTGCATTTAAATAAGAGTGCCTGCAAATTCCTTGGAAAAC[A/T]TTTTATGTTGTTCTACGATTCGAAGGATCGATTAGATCATCAAATGCAATTGTC	Yes	Yes
Polistes SNP #59	scaffold_97:448704	T/C	789	1430	55.2	AAGATATTTGTTTGACGTTTATGGAACAATAATTCTCGTTCTAATTCTAGTCACG[T/C]TACTTGTAATTTATCTTATCAAATTAATAGTGAAATTTGTTTTCGGAAAGAATC		Yes
Polistes SNP #60	scaffold_99:179290	T/C	293	1154	25.4	TAACACCGATGATACCACAGAAGCTTCGTGAGGTGCCGTAACAAAAATGGTCTC[T/C]CCCGAGATTCGATTCATAAATATACAGATCGCTGATTCGATATCGTACATATGT	Yes	Yes
Polistes SNP #61	scaffold_103:395705	A/G	2200	4170	52.8	CTTTCGTAATAATATCAGTGGCAATAGATTACCAACTATCGGTAAAGCACACGGC[A/G]CTGACGGTATACCTTGTCGTTCCCAATAACTATAAATCTGTCGCCAATAAAAAT	Yes	Yes
Polistes SNP #62	scaffold_103:397590	T/A	794	1807	43.9	CCAGTTGAGAAATATTTTTTTGCAATAATAATAATGGTAACAGATGACCAACGAT[T/A]GGCAGGACAAATGGCATCGATGGTATACCTTGCCGTTCCCAATAAGAAAAGAC	Yes	Yes
Polistes SNP #63	scaffold_103:372807	G/A	451	847	53.2	GGCAAGCTGAGGTTCCAGGTTCATCGCTACCGTCCCCGCAATCGCAATAATCATC[G/A]TTGACATAATGAAATGGTATCAATAAGCTACCATCGAGGCATTCAAAATCACGA	Yes	Yes
Polistes SNP #64	scaffold_104:299400	C/T	923	1231	75.0	TCAAATATCACAGGATTCTATCGTAGAATCTTCAATGGGGGTGAAGGAAGGCAAA[C/T]TGAAAGGTTGCATTAAAATATTGGAATGGTTAAATGAATATGAAAGAATTAAT	Yes	Yes
Polistes SNP #65	scaffold_107:235063	T/A	4801	13036	36.8	AGTACTTCAGTGAGGAGCCTGAACTTCTCGAAAGTGTTCTCTACGTCGATAGGAC[T/A]CCTCTTATTTGCGGACAATTCGAAAATGTTCCGAGATATCGAGCCAGGATGCTC	Yes	Yes
Polistes SNP #66	scaffold_111:194174	C/T	688	1208	57.0	ATTCATTGACTTGTGTAACGTTCTAATAGATCCTTAAGTTCTTACTTAATACAAA[C/T]TAGTATCAACATGAATCCGGAGAAGCTTAAGAAACTCCAAGCTCAAGTACGAAT		Yes
Polistes SNP #67	scaffold_120:387511	C/T	6092	10738	56.7	GACATCGTGATCTTGACAAGGATATCATAGATAATATAAAAGCAAAATTGTCAAG[C/T]TATGGAGTAGATCCTTATGATCTTAGTATCATTTCTCAGAGCGGTTGTCCACGC	Yes	Yes
Polistes SNP #68	scaffold_120:392755	C/T	4918	7589	64.8	TTCATGGTATATGTAACAATTCACCTTTCATACGTTCGCATTGCGATTGCGACGC[C/T]AAGTTTCGAAGATGTTTGCAAAATCTTAATACCGAGGTTGCCAACACTATCGGT	Yes	Yes
Polistes SNP #69	scaffold_120:243547	C/G	911	1631	55.9	AAAAACATCTCCACCATCCTAGAATGCAGACCATAAGATTAGCAGCTATACAACA[C/G]GATGCCCATGATAACGATTCGATGAGACCACCGCTATTCTTTTCTCTTCTCCAA	Yes	Yes
Polistes SNP #70	scaffold_120:240573	A/G	504	1601	31.5	CCGCAGCGTCTTCGTCTTGTGAAGTATTAGGGTTATTGCTGTTACTCGACAATGG[A/G]TTGCCAAGTTTGAAACTTCTCTTCCCTTTATTATGCCCATCCTCTTCGTTCCAA	Yes	Yes
Polistes SNP #71	scaffold_122:218647	C/G	3780	7056	53.6	CTTCAGTCTTGCTGACGTGGAAGGGTCTATCTTGAGTCAATTTAGGATCGAACTT[C/G]TTCTTCCAGTTACCCTTGAAGTAGACAGCATTGACAAGCACCATGGCGGTTCCA		
Polistes SNP #72	scaffold_122:197881	C/A	987	2609	37.8	AATGCATTATCAGAGGAGTTGATTTTTATTCCTTGATTTATAAAAGTGGATCAAT[C/A]ACATGCGCGATGAACAGTTCTAATCTCGTATTCGAGGAAATCTTATTTGAGATA		Yes
Polistes SNP #73	scaffold_122:71798	A/G	1150	2219	51.8	TGGCACCAGTTTCTGAATTACTTTTATATTACGTCAGATCAGACGGCGAAACCGT[A/G]GCCACGAGTTATACCATAGAAGTTGGCCATTGTTTCGAAAATAAAGTTAAAACG		Yes
Polistes SNP #74	scaffold_140:103422	T/C	2955	11190	26.4	AAATACTTTTGTTTGGTTTATATAAAGATTTTAATTTAAAATCTAAACGAACGTG[T/C]GTGTGTGTGTTTGTATGTGTTTTACGCTTTATCGTTCGTTAAAGACGTCAGCT	Yes	Yes
Polistes SNP #75	scaffold_140:19142	C/T	284	772	36.8	CTATCAATACCAATTTTAAATTCTTCAATTTCTTGAAATTTTCTTTCGTTATACA[C/T]TCGACTAATTCGTTGTAAGCTACATCAAGTATTTGAAGTTTCGGTAAAAGCTCG	Yes	Yes
Polistes SNP #76	scaffold_143:321740	C/T	268	968	27.7	ACTGATAAATTGATTAGCTAACCGTGAGTGATTTCACTTTATTTTATTTCTCCAA[C/T]ATTTATTTCCTTTCGCGTATTATAATCTCATGTTGGAGAATTCAATCTTAATCA	Yes	Yes
Polistes SNP #77	scaffold_143:180074	A/G	290	939	30.9	ACGGTTGCGCTGAAAAATATTTACGCCTACAAGAATATCCGTTTTCTTCTGAACA[A/G]AAGATGATGGCTGTTAAATGTGCACCCAAATATGACGAAGTAAGCAACGATCTT	Yes	Yes
Polistes SNP #78	scaffold_148:301945	G/A	860	1120	76.8	CCGTCAATTTGTCAATTTCGTTTGTGTTCGAACTTGGACGGACAACGTTGCGTAC[G/A]AAGTTTCCTCTCTCGATTTAAACGATTTTATTATAATATTATTACCTGTGTATG	Yes	Yes
Polistes SNP #79	scaffold_150:366232	G/A	2578	4145	62.2	CTTTCTTATAAACAACTGTGAATCCTTTCTTATCAGGTGTATCCACAATGCCAAC[G/A]CTCTTCTTATGAACTAAACCAGAATACCGGTAGCTACTGAGATTGGTCAAGTTA		Yes
Polistes SNP #80	scaffold_153:105527	G/A	285	1273	22.4	TAATTTTTCTTAACTAGAGGCACTTCTCTTGTTTTCACCTCATCTACAAAACGGA[G/A]TATTTAATCATCGAGACCAAGTCCTTTGGATATTTCTTCTGCCGTCATTTTGTT	Yes	Yes
Polistes SNP #81	scaffold_160:242671	G/T	914	2575	35.5	AGAATGGAACAGGTAGCTCAAGTTCCAGCATAGGTGAGAAATCGAATGGTTCGTC[G/T]CCAACAGGAAAACCAGCAGGAACGCGTACCCGTGTCCCACCTGGTGGATATTCA	Yes	Yes
Polistes SNP #82	scaffold_160:299029	G/A	1431	2099	68.2	TTAATCGATTTATGGATTTAAGAGCATCGGAAAAAGAAAAATTAAAAGACACTGA[G/A]AAATATCAAATTGGTGATGTTACGACAATTAATCTGACTCAATTGAAGGTATAC	Yes	Yes
Polistes SNP #83	scaffold_160:343804	T/C	600	964	62.2	AGATTTTATTTTCCTTATCAGCTTTTATTCTAATAGCTAATTCGGAATTACTGTC[T/C]AAAACATTTTTATCAGTAAGGGACAATAATCTGATTTTATCTAAGGCATCGGAT	Yes	Yes
Polistes SNP #84	scaffold_164:337164	T/C	323	819	39.4	CTCTGAAGGTCGCTTCGGTGGCCGCACTCCACAAGCAACAACAAAATCTTCGTAA[T/C]GCGGTAGAACTCGCCTGGGGCAAAATATTGTCCAGTCACGCACATTTCCCATTA	Yes	Yes
Polistes SNP #85	scaffold_165:266766	T/C	265	1036	25.6	GTTTAAGAGCAGCTAAGAATTTAGTAACCAATGGAATATCCAATTTAGTTGTTA[T/C]GGCGGTGATGGTTCTTTAACTGGTGCTAATCTTTTTAAAGAAGAATGGGCTGAT		Yes
Polistes SNP #86	scaffold_166:311993	A/G	10579	25140	42.1	GTTTAGATTCTTCCGCAGGTTTCGAAGATTTTTCCTCGGATGATTTCTTGTCATC[A/G]TCGTCGTCCTCCTCGCTGTTGTTGTTCTTAGCGTCATCCTTTTCAACAGATTTG	Yes	Yes
Polistes SNP #87	scaffold_166:244932	T/C	366	971	37.7	CTACTTTATTTTTCATAAAATTATCTTCTTGCATGGAAATTTGTGTAAGTTTTTT[T/C]CCTATAGTTCATGTACATCTAAATATTTAGCTACACAACGATCTAAACATATT		Yes
Polistes SNP #88	scaffold_170:196980	T/C	282	938	30.1	ATGTAGCACACATCGAATATCAAACAGAGAAACGTCATTATGGACATACGGATTG[T/C]CCAGGACATGCCGATTATATTAAAAATATGATTACGGGTACTGCACAGATGGAT		
Polistes SNP #89	scaffold_171:309805	C/T	610	2242	27.2	GACCGGAAGATAACAGAGCTGCATGGAGCGGGAAAATGCAATTCTTTCTTACGCAT[C/T]ATTGGTTATAGTGTCGGGCTTGGAAATATTTGGAGGTTTCCTTATCTGTGCCAA	Yes	Yes
Polistes SNP #90	scaffold_171:326870	G/A	580	2058	28.2	CTTACTTTTGGTACAGAACCACCCTGGATGCGGCACCATCAATCGAGGAAGGATC[G/A]TCCCTAAAATGGTGGATCGTTATGTGCCTTTTGCTAAGTTGGATCGTTGTCTTC		Yes
Polistes SNP #91	scaffold_173:80342	T/C	883	2690	32.8	AATGTTAGAGATTAAAAAAAAAAAATTCAATTGATTGGTAGAATCCATTTAAGGAC[T/C]TTCGGGCCACCCTTATACACCATTTTTCCTGATTCTTTTTGATATCACTCCC		
Polistes SNP #92	scaffold_182:118987	G/A	203	796	25.5	TGGGAAATAGAGAATTTGTAATCGACCTTGAAAATACCGAACAAGGTCAGTTGGA[G/A]GCTGATCGTGATAATGCGAATGTTCATAATGGTGTCAATCGTGCTACGGAAAAC		Yes
Polistes SNP #93	scaffold_197:294731	A/C	2114	4590	46.1	GGTTGAACACGAATCCACCAAATATTCCTATATCCTGTTGCAAAGTACAAGAGGA[A/C]TGTAACCCTTTTAATACAGAAAACATTTATACCGAGGTATTGTTTAAACAAATT		Yes
Polistes SNP #94	scaffold_203:102956	G/A	944	3840	24.6	TCCGTCTTCCAATGCATCCTAAATAATATTGTATATCCATTTTCTAAAAACTACC[G/A]ACGCGCTAACTTTTCCTACGAAAAATTGTAAATCAATCTGAATCCGACAAATAT		Yes
Polistes SNP #95	scaffold_206:66144	A/G	602	2370	25.4	TATCGCGTTTACCGCGTTCCCTCGACCGTTCGAAAAATGAGACCAATTGCGATAA[A/G]GTCGCTCTCTTTTTTGAGAACGAGAACAATGTCGTTGCGAGGCTGATTACTGTC	Yes	Yes
Polistes SNP #96	scaffold_206:21319	G/C	367	1255	29.2	ACGCGCTCAACGATATAGAAATTTCATAGCAAAACTTGTGCTCTACGTACATACC[G/C]TTGTATCCACATTTACATTGTATACTGTGATAGTAAAATTTTCGGTGTTACTTT		Yes
Polistes SNP #97	scaffold_208:181331	A/T	193	834	23.1	CGATCGGAAACGATTTTCTAGATTTAACGAAATGTTAAAAGAAAGAAAATAAAT[A/T]AATAAAGAAATGAACAAAAATAAGAATAAGAAGAAGAAGCAAGACAAAAGC		
Polistes SNP #98	scaffold_213:149431	C/T	251	994	25.3	CGTCGATGATATTGACAGGTACTGGATTAGGTGCAAGCATAATCTTCCGTTGCAA[C/T]GATCACATGAAACTAGAAGGTAACGCATCTTCCGTCTGCCAAGCGGAAGGAAAG		
Polistes SNP #99	scaffold_219:27557	C/T	498	902	55.2	TATTCGTTTTCTTTTTGTTTATTATGTGATATAAGTTACGACGATTCGAGTAACT[C/T]AAGCCAATTCGTCGTAGTGTTTGGAAAAACCCAAGAGAAAAAAAACGAAAAAA		
Polistes SNP #100	scaffold_222:77005	T/C	633	1041	60.8	TCGGATAAGAGATTGCTGTTTCGATGTTAATTCGGTTTTTCTTCTTCTTCTTTTT[T/C]TTATCTTTCTTTAACAGGAGAAATTGATCGATCTTCTGAATCGAAAATTTCCTA		Yes

Polistes SNP #101	scaffold_229:217715	A/G	639	2903	22.0	AAGTAACTGGTCATGCTCCTTATGAGAAACGTGCTATGGAATTACTGAAAGTATCA[A/G]AAGGATAAACGCGCATTGAAGTTTTTGAAAAGAAGGGTAAATAAAAAATTTGAT		
Polistes SNP #102	scaffold_260:149328	A/T	208	891	23.3	GAAGAGACCTTATTCTATTTCTCTGCCCCTCAGAGTATCTATAAACCTATCTATT[A/T]ACGTGTATCTATTTAACCGGTTATAATATACTATCAATATTAATTTCTATTCAA	Yes	
Polistes SNP #103	scaffold_262:135330	G/A	4101	8667	47.3	GGTGGCGTGGTCTGCCAACAATGGATGGAAAGACGGCACGAGGTGCATCGTCTCC[G/A]GCAAAACCAGCTTTGCACATACCGGAACCATTGTCAACAACGAGTGCGGCAACT		Yes
Polistes SNP #104	scaffold_266:81083	C/T	912	1965	46.4	GTTACACCTTAAATGCAGCGACTGAAATATGCGAGGACAACGACGAGTGTCTTCT[C/T]GGTACACACGATTGCACCGCAGGCTATCACTGTCGAAATACTATCGGTTCTTAT	Yes	Yes
Polistes SNP #105	scaffold_278:10952	G/A	1537	2451	62.7	AAGAAAAATGATATTTATCTAAAAGTTGATTCGATTTTACCAAAGTTTCGGTGGT[G/A]TGTCATTTATTTATAATTTTCTTCTCCCTGATTGGAATTGTAAAATCGCATAAC		Yes
Polistes SNP #106	scaffold_285:56109	T/C	797	2905	27.4	ACATCATCGTCAAACATCTACGACAATGTTAGGGTCAATCCACTTAATGAATATC[T/C]AGAAAAACAACTTTATACGGTAAACGAACGAACTCGCCAGCTTAATATCATAGA	Yes	Yes
Polistes SNP #107	scaffold_291:159095	T/C	213	939	22.7	TTACCAAATTATTTCCGACTAGATCACATACCGTAGCTGCCCAAGGTGGTATTAA[T/C]GCTGCTTTGGGTAATATGGAAGAAGACAATTGGCAATGGCATATGTATGATACT	Yes	
Polistes SNP #108	scaffold_291:87911	C/T	197	791	24.9	CAGAAACTAAGTTACGCGTAGCCGAGCAACAGCGTAGTAAGTTAGAAGTTGCCAA[C/T]GCGCCACCAGAAAAATTAGCCAGGAGCAAAAAGTATAAGCTCATCGAGAAAGAG	Yes	Yes
Polistes SNP #109	scaffold_292:113025	C/T	253	882	28.7	TTGTAAACGTTACGTCAAATACATACACATATTTCTGAAAAATTACGAAGGTTCT[C/T]TTTTTATCAAATAATTTAAATATAATGCAAATAGCATACGCTGTGGATAATATA	Yes	Yes
Polistes SNP #110	scaffold_312:15152	A/C	640	888	72.1	CAGCTAAACGGGGAGGGGATTTGGGACCATTTAGTAGAGGTACTATGCAGAAACC[A/C]TTCGAGCAAGCAGCATTTGCTTTGAAAGTTGGTGAATTATCTTCTCCTGTCCAT	Yes	
Polistes SNP #111	scaffold_321:148992	G/A	2248	3983	56.4	GAACCAATCGCCTTGCTTCCTGAATTTACGAACGACGCCCGTCCATCGGCACTGA[G/A]CACGGCCTTAAACTTTCGCCCAATCGAAAAAAGAACAAAAATTCGTTTATGTGT	Yes	Yes
Polistes SNP #112	scaffold_321:113115	C/T	655	2425	27.0	TTCTAAATCTACTCTCGAACGGACTGTTGTCATCGTAAATGCTAAATTTGCAATC[C/T]TCCCCATCGGCAATCGAACAGAGGAACGAACCCGGCCTACCATCGGTTTGGGTA	Yes	Yes
Polistes SNP #113	scaffold_322:152232	C/A	689	906	76.0	AACTTTCTGGAATACTCTTGTTCGAAAAAGACAGAGAGAAAGAGAGAAAGAGAAT[C/A]AAACAGACATACAGATAGAAAATCAGACAGACAGACGAATACTATTTAAAATCA	Yes	Yes
Polistes SNP #114	scaffold_351:33861	T/C	469	1987	23.6	CACCGACACTTCCCGAAGACGAACGTATCCCGTTGGATGAAATTAAAGAAGATAT[T/C]GATGAGAAACACGTTATCGAAGAGGTCAAAGAAAAAGATGCGCCTGTTAAAGCT	Yes	
Polistes SNP #115	scaffold_357:80690	C/G	599	1312	45.7	AGAAAAGAACATAGAATAGTTAACGAACGCAAACTTGGAAACTGGGCCAAGCCCA[C/G]CAACATCGCGAGATAACGGATAAAAGAAGAAAAGATGAAATATGCTCGACTCGT		
Polistes SNP #116	scaffold_379:97524	G/A	231	906	25.5	TCAAAATGTTTGCTTTGGAACTTAATGTCACCTATATCTGTCTCTTCGCCACTAA[G/A]TTCCAATTCTGAGGCAACTTCATCACTGCTATTCTCGTCGTGGGATGGCATTAG	Yes	Yes
Polistes SNP #117	scaffold_383:7234	T/C	290	1074	27.0	TTTGTTTTGCTGCACCTCTATTCAATGGAGAATGTTCAAGGCTATCGGTATAATC[T/C]TCGGAATCTTCCGATGCTAATTTTTCTAAACCATGCAATACTTTTGTTCCGATA	Yes	Yes
Polistes SNP #118	scaffold_394:57879	T/C	574	1149	50.0	GAGAACATGGTGTGAGCAAATCAGTTACAATGACGGATGGATCCTTTGGAGATGG[T/C]CCTCTTACTTTTTTGAAGTGGGTGGCTGTTTGAACTTGTCTAACAGGTTGCATG		Yes
Polistes SNP #119	scaffold_411:51860	A/G	606	866	70.0	TTATGCTAGGTCCATCCGCTACATACTCTTCTTCTCCAAGTTTTAACGTTACAGT[A/G]AATCTTTTTTTGTGCGCCGGTCCTTCTTCATTGGTCAGTCTGTATTGATGTTGA	Yes	
Polistes SNP #120	scaffold_426:53026	T/A	12617	20939	60.3	AATTAAGTGTTTTACTGACAGGTGCATCGTTCCAAGAATAAGGTGTTATGTATTG[T/A]CCTATCCATTGATAAATTAATTCATATGCGGTATTCCAAATCAATGAGCTTCTT	Yes	Yes
Polistes SNP #121	scaffold_30:201888	A/T	156	631	24.723	CCATTTTTTTCGTTTTCTCTCGTAAACAACTATTATTTTCGCTTAAAATTTATA[A/T]TCTGCTGCTTTCCGCGCATTTCGCAGTTCGCGACAGGTGATCGATGGATTTCT		

Appendix III


Percentage of Patrilines per Matriline in Offspring
0
20
40
60
80
100
6 8 3 7 5 7 19 8 7 6 11 12 6 6 7 8 6 5 9 8 8 6 6 8 5 5 5

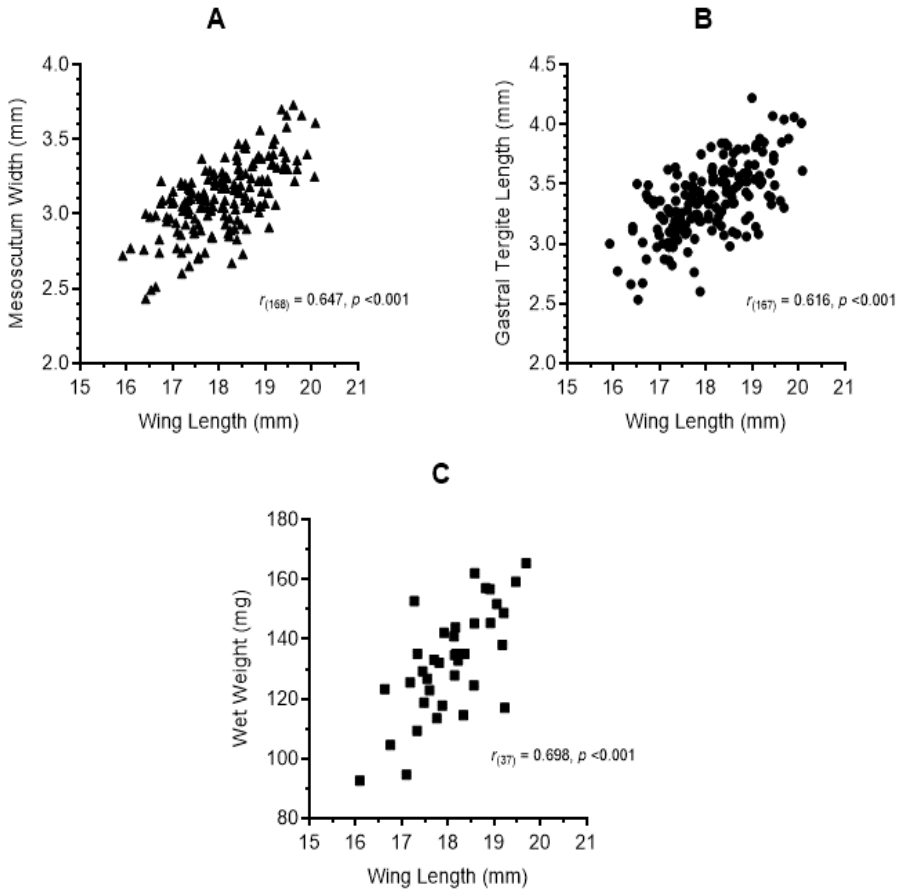
Appendix Table 3. Converted section of output from COLONY 2.0.6.3 (Jones & Wang, 2010), in examining occurrences of multiple mating in P. lanio (Chapter 3). Normal Output: adults assigned as potential mothers to pupae, any adults that do not have maternity in pupae re-assessed as potential offspring. Output if Mother/Offspring Structure Assignment Ignored (Hypothetical): all adults and pupae assigned as potential offspring. Clustering probability 0 to 1 (1 = 'certain'). Numbers in Paternity/Maternity assignment represent non-genotyped fathers and mothers.

Normal Output				Output if Mother/Offspring Structure Assignment Ignored (Hypothetical)				
Probability	**Sample**	**Paternity**	**Maternity**	**Probability**	**Sample**	**Paternity**	**Maternity**	
0.9987	Nest #04 Forager	4	Nest #08 Egglayer	0.9922	Nest #08 Egglayer	6	4	
0.9987	Nest #04 Pupae 5	13	Nest #08 Egglayer	0.9922	Nest #04 Forager	6	4	← *still estimates separate fathers for the forager and pupae*
0.9987	Nest #04 Pupae 4	13	Nest #08 Egglayer	0.9922	Nest #04 Pupae 5	20	4	
0.9987	Nest #04 Pupae 3	13	Nest #08 Egglayer	0.9922	Nest #04 Pupae 4	20	4	
0.9987	Nest #04 Pupae 2	13	Nest #08 Egglayer	0.9922	Nest #04 Pupae 3	20	4	
0.9987	Nest #04 Pupae 1	13	Nest #08 Egglayer	0.9922	Nest #04 Pupae 2	20	4	
1.0000	Nest #02 Forager	7	Nest #02 Egglayer	0.9922	Nest #04 Pupae 1	20	4	
1.0000	Nest #02 Pupae 5	14	Nest #02 Egglayer	0.9928	Nest #02 Egglayer	11	7	
1.0000	Nest #02 Pupae 4	14	Nest #02 Egglayer	0.9928	Nest #02 Forager	12	7	← *still estimates separate fathers for the forager and pupae*
1.0000	Nest #02 Pupae 3	14	Nest #02 Egglayer	0.9928	Nest #02 Pupae 5	21	7	
1.0000	Nest #02 Pupae 2	14	Nest #02 Egglayer	0.9928	Nest #02 Pupae 4	21	7	
1.0000	Nest #02 Pupae 1	14	Nest #02 Egglayer	0.9928	Nest #02 Pupae 3	21	7	
0.7553	Nest #08 Egglayer	11	3	0.9928	Nest #02 Pupae 2	21	7	
0.7553	Nest #08 Pupae 5	15	3	0.9928	Nest #02 Pupae 1	21	7	
0.7553	Nest #08 Pupae 4	15	3	0.6783	Nest #08 Egglayer	15	9	← *still estimates separate fathers for the egglayer and pupae*
0.7553	Nest #08 Pupae 3	15	3	0.6783	Nest #08 Pupae 5	22	9	
0.7553	Nest #08 Pupae 2	15	3	0.6783	Nest #08 Pupae 4	22	9	
0.7553	Nest #08 Pupae 1	15	3	0.6783	Nest #08 Pupae 3	22	9	
0.8913	Nest #08 Forager	9	2	0.6783	Nest #08 Pupae 2	22	9	
				0.6783	Nest #08 Pupae 1	22	9	
				0.5732	Nest #08 Forager	16	10	

Appendix V

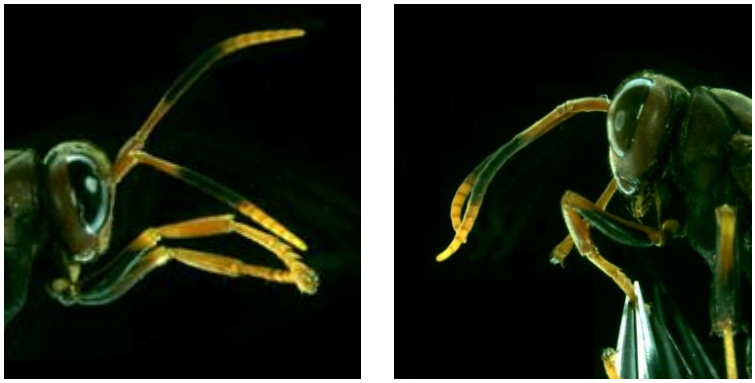
Appendix Figure 2. Individual M1-25, the oldest recorded *P. canadensis* at 506 days old.

Appendix VI



Appendix Figure 3. Relationship between male *P. lanio* wing length (mm) against: (A) mesoscutum width (mm); (B) gastral tergite length (mm); and (C) wet weight of newly emerged males. Pearson's correlations, α = 0.05.

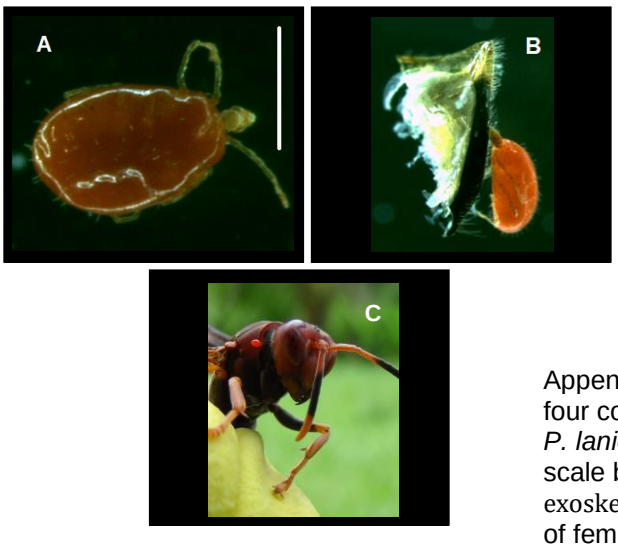
Appendix VII



Appendix Figure 4. Example of male eye colouration change in *P. lanio*. On the left, a newly emerged male; on the right, a 10 day old male.

Appendix VIII

Four unidentified mites (presumed conspecifics) were discovered on a dispersed *P. lanio* male (non-nesting) caught at site Verdant Vale (VV) (Chapter 5). These mites were also rarely discovered on females (pers. obs.) at sites Verdant Vale (VV) and Eastern Main Rd (EM) (Appendix Figure 5). It was unknown whether these were parasitic mites or engaged in phoresy. The male with discovered mites, had no visible difference in testes area or seminal vesicles and accessory gland fluid area compared to other dispersed individuals.



Appendix Figure 5. Unidentified mite (one of four conspecifics) discovered on a dispersed *P. lanio* male: (A) dorsal-profile with 0.5 mm scale bar; (B) lateral-profile attached to wasp exoskeleton; mite (C) attached to exoskeleton of female *P. lanio* forager.

Appendix IX

Alternative zero-inflation generalized linear mixed models using R 3.3.3 (R Core Team, 2017) and 'glmmTMB' package (Brooks et al., 2017), for Chapter 6. Zero-inflated generalized linear models fitted with the package 'glmmTMB' allow for separate outputs of the binary and poisson distribution: the binary output of the model being the difference between events (i.e. occurrences happening) and structural-zeros; the poisson distribution being the fit of sampled-zeros and variation in counts.

Model 1 Methodology: To assess the occurrence of male helping within the context of either gaining food from foraging females in *natural trials* or accepting experimental food during *food-offering trials*, we analysed male–cell interaction as counts with a zero-inflated GZLMM (poisson distribution), using the simplified fixed effect of food state (four levels: *natural trial* – no food gained from foraging females; *natural trial* – food gained from foraging females (combining food gained, and lost to another adult wasp); *food-offering trial* – accepted experimental food-offering (combing whether additional food was gained from a foraging female and/or if any food was lost to another adult wasp); *food-offering trial* – rejected experimental food-offering). The model accounted for multiple repeated measures on the same individuals, by fitting trial number by wasp ID as a random effect term.

Model 2 Methodology: We analysed male–cell interactions in *food-offering trials* in relation to age. We ran a zero-inflated GZLMM (poisson distribution), with the response variable of how many interactions a focal male had with cells containing larvae within the 25 min period after food was offered. The fixed effects for the model were the response to the food offering (whether it accepted or rejected the offering) and male age (day). The model accounted for multiple repeated measures on the same individuals, by fitting trial number by wasp ID as a random effect term.

Model 1 Results: Using *natural trials* in which males received no food from foraging females as a baseline… (1) males that gained food from returning foraging females in other *natural trials* had significantly more occurrences of male-cell interactions (Binary: Z-value = 2.76, p = 0.006), but the count of male-cell interactions did not change when interactions happened (Poisson: Z = -0.07, p = 0.941); (2) males that accepted experimental food offerings in *food-offering trials* had significantly more occurrences of male-cell interactions (Binary: Z = 1.95, p = 0.05), but again the count of male-cell interactions did not change when interactions happened (Poisson: Z = -0.68, p = 0.499); (3) males that rejected experimental food offerings in *food-offering trials* did not have any significant change in male–cell interactions (Binary: Z = -0.238, p = 0.812; Poisson: Z = 0.215, p = 0.830); (4) Random effect variance of individual ID on the binary model was 1.75 (SD = 1.32). *Note* – lack of significant differences in the poisson distribution likely represent that age is not yet factored into the model as a fixed effect.

Model 2 Results: Within *food-offering trials*… (1) of males that accepted food, younger males had significantly more occurrences of male-cell interactions than older males (Binary: Z-value = -5.08, $p < 0.001$), and of those males that did interact with brood, younger males had more counts of male–cell interactions than older males (Poisson: Z = -6.94, $p = 0.001$); (2) of trials in which males rejected food, there was no effect of age on whether males had more/less occurrences of male-cell interactions (Binary: Z-value = -1.38, $p = 0.168$), however of those that did interact with brood, older males had more male-cell interaction counts than younger males (Poisson: Z = 2.52, $p = 0.012$). *Note* – this last poisson distribution of 'reject' males contains only a single male that had one interaction, when this male is removed from the model, then the distribution is void (all remaining 'reject' males have a value of zero counts). Random effect variance of trial and individual ID on the model was: Binary Model 0.28 (SD = 0.53); Poisson Distribution: 0.64 (SD = 0.80).